Unclassified

AD_____

REPORT NUMBER FIVE

GENETIC AND PHYSIOLOGICAL CONTROL OF PROTECTIVE ANTIGEN SYNTHESIS BY BACILLUS ANTHRACIS

ANNUAL PROGRESS REPORT

CURTIS B. THORNE

DECEMBER 1984

Supported by

U. S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701

Contract No. DAMD 17-80-C-0099

University of Massachusetts
Amherst, Massachusetts 01003

APPROVED FOR PUBLIC RELEASE

DISTRIBUTION UNLIMITED

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

Unclassified

Unclassified

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE	READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER	2. GOVT ACCESSION NO. A156 324 — 3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) Genetic and Physiological Control of Protective Antigen Synthesis by Bacillus anthracis	5. TYPE OF REPORT & PERIOD COVERED Annual Report Jan. 1, 1984-Dec. 31, 1984 6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) Curtis B. Thorne	8. CONTRACT OR GRANT NUMBER(s) DAMD 17-80-C-0099
9. PERFORMING ORGANIZATION NAME AND ADDRESS Department of Microbiology University of Massachusetts Amherst, MA 01003	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 61102A.3M161102BS12.AB.060
11. CONTROLLING OFFICE NAME AND ADDRESS U.S. Army Medical Research and Development Command Fort Detrick, Frederick, MD 21701	12. REPORT DATE December 1984 13. NUMBER OF PAGES 48
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office)	15. SECURITY CLASS. (of this report) Unclassified 15a. DECLASSIFICATION/DOWNGRADING SCHEDULE
16. DISTRIBUTION STATEMENT (of this Report) Approved for public release; distribution unlimited	
17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)	
18. SUPPLEMENTARY NOTES	
19. KEY WORDS (Continue on reverse side if necessary and identify by block number) Bacillus anthracis, Anthrax protective antigen, Anthrax toxin, B. anthracis plasmid.	

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

--The primary objective of the research is to gain information and to develop genetic systems that will contribute to the development of an improved vaccine for anthrax. Genetic and physiological factors controlling the synthesis and accumulation of protective antigen as well as the two other components of anthrax toxin are being investigated. During the past year attention was focused on (1) further development and application of a mating system for transfer of plasmids among Bacillus species, and (2) studies of a

DD FORM 1 JAN 73 1473 EDITION OF 1 NOV 65 IS OBSOLETE

Unclassified

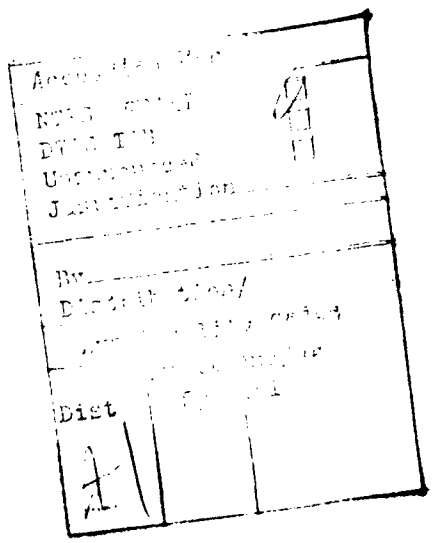
SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

newly discovered plasmid which encodes capsule formation in B. anthracis.

The mating system for transfer of plasmids among B. anthracis, B. cereus, and B. thuringiensis is very useful for assessing the biological significance of plasmids found in the three species. We have identified two B. thuringiensis plasmids, pXO11 and pXO12, which are capable of bringing about their own transfer as well as that of other plasmids. B. anthracis and B. cereus transcipients that inherit either pXO11 or pXO12 were, in turn, effective donors. By means of the mating system we have been able to demonstrate transfer of the anthrax toxin plasmid, pXO1, to B. cereus and strains of B. anthracis previously cured of the plasmid.

We have demonstrated that the avirulent Pasteur strain (ATCC 6602) of B. anthracis carries a plasmid, pXO2, which encodes capsule formation. In collaboration with Dr. Bruce Ivins of USAMRIID we have shown that virulent strains of B. anthracis also contain pXO2. Two classes of rough noncapsulated variants were isolated from the capsule-producing pasteur strain; those which were cured of pXO2 and those which still carried the plasmid. Reversion to capsule formation was observed only in the variants that retained pXO2. The Weybridge (Sterne) strain does not contain pXO2 and that explains why capsule-forming revertants have not been observed with that strain.

By means of the Bacillus mating system we have transferred the capsule plasmid, pXO2, to B. cereus. B. cereus transcipients that acquired the plasmid produced capsules under the same conditions required for capsule synthesis by B. anthracis.



Unclassified

AD_____

REPORT NUMBER FIVE

GENETIC AND PHYSIOLOGICAL CONTROL OF PROTECTIVE ANTIGEN SYNTHESIS BY *BACILLUS ANTHRACIS*

ANNUAL PROGRESS REPORT

CURTIS B. THORNE

DECEMBER 1984

Supported by

U. S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701

Contract No. DAMD 17-80-C-0099

University of Massachusetts
Amherst, Massachusetts 01003

APPROVED FOR PUBLIC RELEASE

DISTRIBUTION UNLIMITED

Unclassified

SUMMARY

This is a progress report (annual report) of research being carried out with Bacillus anthracis. The primary objective of the research is to gain information and to develop genetic systems that will contribute to the development of an improved vaccine for anthrax. The toxin-producing, but avirlent, Weybridge (Sterne) strain of B. anthracis is being used. Genetic and physiological factors controlling the synthesis and accumulation of protective antigen as well as the two other components of anthrax toxin are being investigated. During the past year our major effort was placed on further development and application of a mating system by means of which plasmids can be transferred among strains of B. anthracis, B. cereus, and B. thuringiensis. We also spent considerable effort on studies of a newly discovered plasmid which encodes capsule formation in B. anthracis.

The mating system for transfer of plasmids among B. anthracis, B. cereus, and B. thuringiensis is very useful for assessing the biological significance of plasmids found in the three species. The tetracycline resistance plasmid, pBC16, which we moved into B. thuringiensis by transduction, made it possible to select transcipients quantitatively. We have identified two B. thuringiensis plasmids, pXO11 and pXO12, which are capable of bringing about their own transfer as well as that of pBC16 and other plasmids. B. anthracis and B. cereus transcipients inheriting either pXO11 or pXO12 were, in turn, effective donors. Transcipients harboring pXO12 were more efficient donors than those harboring pXO11; transfer frequencies ranged from 10^{-4} to 10^{-1} and form 10^{-8} to 10^{-5}, respectively. Cell-to-cell contact was necessary for plasmid transfer and the addition of deoxyribonuclease to mating mixtures had no effect. The high frequencies of transfer, along with the fact that cell-free filtrates of donor cultures were ineffective, suggested that the transfer was not phage-mediated. B. anthracis and B. cereus transcipients that inherited pXO12 also acquired the ability to produce parasporal crystals (Cry^+) resembling those produced by B. thuringiensis, indicating that pXO12 carries gene(s) involved in crystal formation. Transcipients that inherited pXO11 were Cry^-. This mating system provides an efficient method for transfer of a large range of Bacillus plasmids within and among species by a conjugation-like process.

By means of the mating system we have been able to demonstrate transfer of the anthrax toxin plasmid, pXO1, to strains of B. anthracis previously cured of

the plasmid. Transcipients that inherited pXO1 regained the ability to synthesize protective antigen and, in addition, they resembled the original uncured parent strain with respect to a number of other phenotypic characteristics. This provides proof that the phenotypic changes observed in cured strains were, in fact, a result of plasmid loss rather than a consequence of the selection procedure used in their isolation.

To determine whether pXO1 had integrated into the bacterial chromosome of strains presumed to be cured of the plasmid, and also to explore the possibility that pXO1 and the chromosome might have sequences in common, we carried out hybridization experiments using pXO1 DNA as a probe. Results indicate that pXO1 was completely absent from the cured strains and also that the plasmid does not share homologous sequences with the chromosome.

Very recently we have demonstrated transfer of pXO1 to B. cereus at a high frequency; more than 50% of tetracycline-resistant transcipients also inherited pXO1. It will be important to study the biological activity of this plasmid in B. cereus.

We have demonstrated that the avirulent Pasteur strain (ATCC 6602) of B. anthracis carries a plasmid, pXO2, which encodes capsule formation. In collaboration with Dr. Bruce Ivins of USAMRIID we have shown that virulent strains of B. anthracis also contain pXO2. Two classes of rough noncapsulated (Cap^-) variants were isolated from the capsule-producing (Cap^+) Pasteur strain; those which were cured of pXO2 and those which still carried it. Reversion to Cap^+ was demonstrable only in rough variants which contained pXO2. This explains an observation reported several years ago that some Cap^- variants of B. anthracis were revertible to Cap^+ and others were nonrevertible. By means of the mating system discussed above, pXO2 was transferred from B. anthracis to B. cereus. B. cereus transcipients which acquired pXO2 produced capsules under the same conditions required for capsule synthesis by B. anthracis.

Foreword

Citation of trade names in this report does not constitute an official Department of the Army endorsement or approval of the use of such items.

TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

This is the fifth annual report submitted under contract No. DAMD 17-80-C-0099. Research on the contract began July 1, 1980. The contract was extended for a second year beginning July 1, 1981, for two more years on July 1, 1982, and it was finally extended a third time for a fifth year beginning August 1, 1984. The previous annual reports are dated December 1980, December 1981, December 1982, and December 1983.

During the year represented by this annual report our research concentrated largely on aspects of a mating system for the transfer of plasmids among B. anthracis, B. cereus, and B. thuringiensis. As a result we have been able to transfer B. anthracis plasmids to B. cereus and cured strains of B. anthracis. This makes it possible to better evaluate the biological significance of the plasmids in question. We have demonstrated during the past year that capsule synthesis by B. anthracis is encoded by a newly discovered plasmid, which we have designated pXO2. In this report our main efforts for the past year are discussed following a general description of materials and methods. Specific procedures which themselves are results of the research are described as appropriate under individual sections.

MATERIALS AND METHODS

Organisms. The Weybridge (24) strain of B. anthracis was obtained from the Microbiological Research Establishment, Porton, England in 1957. It was isolated by Sterne (21) and used by the Ministry of Agriculture, Fisheries, and Food (Weybridge, England) as a living spore vaccine. Table 1 lists specific strains and mutants referred to in this report.

Media. For convenience to the reader, compositions of the various culture media commonly used in our laboratory are given below. All amounts are for one liter final volume. For preparation of solid medium, 15 grams of agar (Difco) were added per liter of the corresponding broth.

NBY broth: Nutrient broth (Difco), 8 g; Yeast extract (Difco), 3 g.

Phage assay broth: Nutrient broth (Difco), 8 g; NaCl, 5 g; $MgSO_4.7H_2O$, 0.2 g; $MnSO_4.H_2O$, 0.05 g; $CaCl_2.2H_2O$, 0.15 g. The pH was adjusted to 6.0 with HCl.

Phage assay agar: For bottom agar, 15 g of agar were added per liter of phage assay broth. For soft agar, 0.6 g of agar were added per liter.

L broth: Tryptone (Difco), 10 g; Yeast extract (Difco), 5 g; NaCl, 10 g. The pH was adjusted to 7.0 with NaOH.

BHI broth: Brain heart infusion broth (Difco), 37 g.

BHI-glycerol broth: BHI broth with 0.5% (w/v) glycerol added aseptically.

Minimal I: $(NH_4)_2SO_4$, 2 g; KH_2PO_4, 6 g; K_2HPO_4, 14 g; sodium citrate, 1 g; glucose, 5 g; L-glutamic acid, 2 g; $MgSO_4.7H_2O$, 0.2 g; $FeCl_3.6H_2O$, 0.04 g; $MnSO_4.H_2O$, 0.00025 g. The pH was adjusted to 7.0 with NaOH. The glucose and $FeCl_3$ were sterilized separately.

Minimal IC: Minimal I with 5 g of vitamin-free Casamino acids (Difco) and 10 mg of thiamine hydrochloride.

Minimal M: To Minimal I was added 10 mg of thiamine hydrochloride, 200 mg of glycine, and 40 mg of L-methionine and L-proline.

Minimal O: To Minimal I was added 10 mg of thiamine hydrochloride, 200 mg of glycine, and 40 mg of L-methionine, L-serine, L-threonine, and L-proline.

Antisera. All antisera were kindly supplied by personnel of USAMRIID.

Protective antigen assays. These were carried out by the agar diffusion method as outlined by Thorne and Belton (24).

Propagation and assay of bacteriophage CP-51. The methods described by Thorne (23) were followed. The indicator for routine assay of CP-51 was B. cereus NRRL B-569.

Propagation and assay of bacteriophage Wα. Bacteriophage Wα (12) was obtained from B. cereus W (ATCC 11950). It was propagated on B. anthracis 6602 R1 in soft overlays of phage assay agar incubated at 37°C for 17 to 20 hours. It was assayed against the same strain in soft overlays of phage assay agar incubated at 30°C.

Test for capsule production. The ability of B. anthracis and B. cereus to produce capsules was determined by growing cells on R agar or on NBY agar supplemented with 0.7% (w/v) sodium bicarbonate and 10% (v/v) horse serum. Plates were incubated in the presence of 20% CO_2 at 37°C for 24 to 48 hours.

Isolation of spontaneous rough mutants of B. anthracis 6602. Cells were plated for single colonies on NBY agar containing 0.7% (w/v) bicarbonate and 10%

(v/v) horse serum and incubated at 37°C in 20% CO_2. After several days rough outgrowth appeared at the edge of some of the mucoid colonies. These were picked and purified by streaking on fresh plates of the same medium.

Isolation of capsulated revertants of rough mutants. To demonstrate reversion, approximately 1 x 10^5 spores of a rough mutant were spread with 1 x 10^8 PFU of phage Wα on NBY agar containing bicarbonate and horse serum as above. The plates were incubated at 37°C in 20% CO_2 for two days and examined for mucoid colonies. The presence of capsules was confirmed by phase microscopy.

Procedures used in mating experiments. Cells for mating were grown in 250-ml Erlenmeyer flasks containing 25 ml of BHI broth and incubated at 30°C with slow shaking. Donor and recipient strains were grown separately for 8 to 10 hours from 1% (v/v) transfers of 14- to 15-hour cultures. Each culture was diluted 1:50 in BHI broth, yielding 10^6 to 10^7 cells per ml, and mating mixtures were prepared by mixing 1 ml of donor cells with 1 ml of recipient cells in 20-mm culture tubes. Control tubes contained 1 ml of BHI broth and 1 ml of donor or recipient cells. Mixtures were incubated at 30°C with slow shaking. Samples were removed at times indicated and plated on appropriate selective media for determining the numbers of donors, recipients, and transcipients. Dilutions were made in peptone diluent. Plates were incubated at 30°C and colonies were scored after 24 to 48 hours.

When mating mixtures were prepared with streptomycin-resistant recipients and tetracycline-resistant donors, tetracycline-resistant transcipients were selected on L-agar containing streptomycin (200 µg/ml) and tetracycline (5 or 25 µg/ml. If the recipients were streptomycin-sensitive, tetracycline-resistant transcipients were selected on Min 1C agar supplemented with tetracycline and the appropriate growth requirement of the auxotrophic recipient. For selecting B. cereus transcipients 25 µg of tetracycline per ml was used, but with B. anthracis the number of transcipients recovered was greater when the concentration of tetracycline was only 5 µg per ml. Once transcipients were selected with the lower concentration of tetracycline, they were then fully resistant to 25 µg per ml. When recipients were rifampicin-resistant, rifampicin (10µg/ml) was included in the selection medium.

Transfer frequency is expressed as the number of transcipients per ml divided by the number of donors per ml at the time of sampling. It should be emphasized that the use of both auxotrophic and drug-resistant strains allowed unambiguous strain selection and recognition.

Test for effect of DNase in mating mixtures. Donor cells were first incubated alone in the presence of 100 µg of DNase per ml (Worthington Biochemical Corp.) and 0.01 M $MgSO_4$ for 15 min at 37°C. One ml of donor and 1 ml of recipient cells were mixed together, and DNase (100 µg/ml) was added again after 1, 2, and 3 hours of mating. $MgSO_4$ without DNase was added to control matings. After 4 hours of incubation samples were plated for selection of transcipients.

Test for plasmid transfer by bacteriophage in mating mixtures. To investigate the possibility of pnage-mediated plasmid transfer, cell-free filtrates of donor cultures were substituted for donor cells. The supernatant fluid from a centrifuged donor culture was filtered through a Millipore HA membrane (Millipore Corp., Bedford, MA), and 1 ml of cell-free filtrate was mixed with 1 ml of recipient cells. Such mixtures were incubated and assayed for Tc^r transcipients as described above.

Detection of plasmid DNA. Plasmid DNA was extracted by a modification of the procedure described by Kado and Liu (9). Cells for plasmid extraction were grown in 250-ml Erlenmeyer flasks containing 25 ml of BHI broth supplemented when appropriate with tetracycline (10 µg/ml). With some strains better results were obtained when 0.5% (w/v) glycerol was included in the BHI broth to prevent sporulation. Cultures were incubated for 16 hours at 37°C on a rotary shaker (100 to 160 rpm). Cells from 25 ml of culture were collected by centrifugation at 10,000 rpm in a Sorvall SS34 rotor for 10 min at 15°C and resuspended in 1 ml of E buffer (0.04 M Tris-OH (Sigma), 0.002 M EDTA (tetrasodium salt, Sigma), 15% sucrose, pH 7.9) by gentle vortexing. Cells were lysed by adding 1 ml of the suspension to 2 ml of lysis buffer prepared by adding 3 gm of sodium dodecyl sulfate and 5 ml of 3 N NaOH to 100 ml of 15% (w/v) sucrose in 0.05 M Tris-OH. The tubes were rapidly inverted 20 times to mix the cells and buffer and they were then held in a 60°C water bath for 30 min. Five-tenths ml of Pronase (Calbiochem-Behring Corp., La Jolla, CA) solution (2 mg per ml in 2 M Tris at pH 7.0) was added, and the tubes were mixed as above and incubated in a 37°C water bath for 20 min. The lysate was extracted with 6 ml of phenol-chloroform (1:1, v/v) by inverting the tubes 40 times. The emulsions were separated by centrifugation at 10,000 rpm for 10 min at 15°C and the aqueous phase was removed for electrophoresis.

Extracts (40 µl) were mixed with 10 µl of tracking dye (0.25% bromphenol blue, 15% ficoll) and samples (40 µl) were applied to horizontal 0.7% agarose

(Sigma, Type II medium EEO) gels prepared and run in Tris-borate buffer (0.089 M Tris-OH, 0.089 M boric acid, 0.0025 M EDTA, pH 8.2 to 8.3). Electrophoresis was carried out at 70 V for 90 to 120 min at room temperature. Gels were stained with ethidium bromide (1 µg/ml in Tris-borate buffer).

Screening colonies for protective antigen production. Colonies were picked to plates of R medium (14, 17) and incubated at 37°C in 20% CO_2. Each "halo agar" plate contained 12 ml of R agar plus 2 ml of antiserum prepared in goats by immunization with viable spores of the Sterne strain of B. anthracis. A zone of precipitate formed around PA^+ colonies.

RESULTS AND DISCUSSION

I. Renaming of B. anthracis and B. thuringiensis plasmids

When my laboratory began work on plasmids we chose the designation pBA for plasmids of B. anthracis and pUM or pBT for plasmids of B. thuringiensis. However, it came to our attention that these three designations were already in use by other laboratories. Therefore, we have now chosen the designation pXO for all plasmids named by our laboratory. This designation has been approved and officially assigned to our laboratory by the Plasmid Reference Center at Stanford University. Accordingly, the B. anthracis toxin plasmid, which was originally designated pBA1, is now named pXO1. The plasmid found in the Pasteur strain of B. anthracis (ATCC 6602) and associated with capsule formation is named pXO2. The B. thuringiensis fertility plasmid formerly referred to as pUM1 or pBT1 is now named pXO11, and the fertility plasmid formerly referred to as pUM2 or pBT2 is now named pXO12.

II. Plasmid profiles of the Pasteur strain (ATCC 6602) and the M strain of B. anthracis

The Pasteur strain (ATCC 6602) of B. anthracis carries one large plasmid, which we have designated pXO2. The plasmid migrates more rapidly than pXO1 during electrophoresis in agarose gels, and we estimate that its molecular mass is in the order of 60 megadaltons. As discussed later in this report, we have shown that pXO2 is involved in capsule formation.

The M strain of B. anthracis, a mutant that does not require added bicarbonate for the synthesis of capsules, carries two plasmids which migrate in agarose gels at the same rates as pXO1 and pXO2. This observation has stimulated our interest in the role of bicarbonate in synthesis of capsule. Bicarbonate is required for the synthesis of capsules by the Pasteur strain, 6602, and by virulent strains. It will be interesting to learn whether the mutation which enables the M strain to produce capsules in the absence of bicarbonate is carried by the plasmid, pXO2, or whether it is chromosomal. We should be able to determine this by transferring pXO2 from the M strain to other strains of B. anthracis that have been cured of pXO2. If the mutation is on the plasmid, strains infected with the mutant plasmid should not require bicarbonate for capsule production. On the other hand, if the mutation is chromosomal, strains infected with the plasmid should not have the mutant phenotype. We hope to answer this question in the near future by using the mating system to transfer pXO2 from the M strain to appropriate recipients.

III. The mating system for transfer of plasmids among strains of B. anthracis, B. cereus, and B. thuringiensis

We have continued to investigate the mating system described in Report Number Four (Annual Progress Report, December 1983). We have concentrated our studies on two fertility plasmids found in B. thuringiensis 4042A subsp. thuringiensis. The following summarizes the utility of the mating system and the current status of our understanding of the process.

Transfer of pBC16 from B. thuringiensis subsp. thuringiensis. Plasmid profiles of the two donor strains, B. thuringiensis 4042A UM8 td2 and 4042A UM8-13 td1-A, as well as B. anthracis and B. cereus recipients and transcipients are shown in Fig. 1 and 2. All Tc^r transcipients inherited plasmid DNA which migrated at the same rate as pBC16. In addition to pBC16, most transcipients also inherited various combinations of other plasmids derived from the B. thuringiensis donor. Examination of a large number of transcipients has shown that (1) the two most frequently transferred B. thuringiensis plasmids were pXO11 and pXO12 and (2) the smaller molecular weight plasmids were transferred more or less randomly as demonstrated by their variable distribution in transcipients. In matings with donor strain 4042A UM8-13 td1-A, which harbors

pXO11 as well as several other unnamed plasmids, the majority of Tc^r transcipients acquired pXO11. Similarly, with donor cells of 4042A UM8 td2, which harbors both pXO11 and pXO12, the majority of Tc^r transcipients inherited either pXO11 or pXO12. However, we have observed that in matings with the latter donor, transcipients inherited pXO11 more frequently than pXO12. No transcipients thus far examined have contained both of these plasmids, suggesting that there may be competition between pXO11 and pXO12 during transfer. All transcipients retained both the auxotrophic and streptomycin resistance markers of the recipient strain. Although spontaneous tetracycline-resistant mutants of B. cereus 569 were occasionally found at low frequencies, we have never observed such spontaneous mutants of B. anthracis.

Formation of parasporal crystals by B. anthracis and B. cereus transcipients. Phase microscopy revealed that some Tc^r transcipients derived from matings in which strain 4042A UM8 td2 was the donor had also acquired the ability to produce parasporal crystals (Cry^+) resembling those produced by the B. thuringiensis donor. In contrast, no Cry^+ transcipients were obtained from matings in which strain 4042A UM8-13 td1-A was the donor. Plasmid analysis of the two donor strains indicated that pXO12 was associated with crystal production. As shown in Fig. 2, (lanes 1 and 6), the plasmid profiles of the Cry^- mutant, 4042A UM8-13 td1-A, and the Cry^+ mutant, 4042A M8 td2, were identical except for the absence (spontaneous loss, see footnote i to Table 1) of pXO12 from UM8-13 td1-A. This, along with the fact that there was a strict correlation between the Cry^+ phenotype and the presence of pXO12 in transcipients, is strong evidence that pXO12 is involved in crystal production. A number of reports have established that one or more plasmids are involved in parasporal crystal formation in a variety of B. thuringiensis strains (3-8, 10, 11, 15, 19, 20). Although Cry^+ transcipients of both B. anthracis and B. cereus were obtained from matings in which 4042A UM8 td2 was the donor, the frequency of Cry^- transcipients was 10^2 to 10^3 times higher than that of Cry^+ transcipients. The lower frequency of Cry^+ transcipients is in agreement with plasmid analyses which showed that the donor, 4042A UM8 td2, transferred pXO12 much less frequently than pXO11.

Transfer of pBC16 and other plasmids from B. anthracis and B. cereus transcipients. In our system of labeling transcipients for identification, e.g., Weybridge UM44 tr203-1, UM44 designates the recipient from which the transcipient was derived and tr203-1 identifies a particular transcipient that

has been purified by single colony isolation. Transcipients isolated from mating mixtures in which B. thuringiensis was the donor are referred to as primary transcipients. Secondary transcipients are those derived from matings in which the donors were fertile B. cereus or B. anthracis transcipients harboring either pXO11 or pXO12.

Matings were performed to determine whether primary and secondary B. anthracis and B. cereus transcipients could also function as donors of pBC16 to tetracycline-sensitive B. anthracis and B. cereus recipients. Results (Table 2) show that B. anthracis and B. cereus transcipients which acquired either pXO11 or pXO12 were, in turn, effective donors of pBC16. Plasmid analysis confirmed the transfer of pBC16 as well as other B. thuringiensis plasmids from the primary and secondary transcipients. Transcipients which inherited only the smaller B. thuringiensis plasmids migrating below chromosomal DNA (Fig. 1, lane 8) were not fertile. Donor ability of the fertile transcipients was stably maintained during subsequent growth and sporulation. pBC16, itself, was ineffective in promoting plasmid transfer; this was evidenced by the fact that cells of B. anthracis or B. cereus into which pBC16 was introduced by transduction were completely devoid of donor activity.

As shown in Table 2, transcipients that harbored pXO12 were more effective donors of pBC16 than those that harbored pXO11. The data there also reflect a difference between B. cereus and B. anthracis with respect to their activity as recipients. In experiments with $(pXO12)^+$ transcipients as donors, B. cereus recipients usually yielded 10- to 100-fold more transcipients than B. anthracis recipients. However, with $(pXO11)^+$ transcipients as donors, recipient cells of the two species yielded approximately equal numbers of transcipients.

Tc^r transcipients from both intraspecies and interspecies matings retained the auxotrophic marker of the recipient strain. Prototrophic recombinants were never found, suggesting that transfer of chromosome occurred rarely or not at all.

Evidence for plasmid mobilization by pXO11 and pXO12. Taking advantage of a large collection of B. anthracis and B. cereus transcipients displaying various plasmid profiles, we attempted to identify the fertility factors responsible for plasmid mobilization. Based on the fact that all transcipients harboring either pXO11 or pXO12 were capable of plasmid transfer, we speculated that either of these two plasmids alone could confer donor capability to host cells. Examination of plasmid content and transfer ability of numerous Tc^r

transcipients confirmed that both pXO11 and pXO12 are fertility plasmids, each capable of promoting its own transfer as well as that of other plasmids. The random distribution of the smaller B. thuringiensis plasmids in fertile transcipients suggested that no combination of these plasmids in conjunction with either pXO11 or pXO12 was necessary for plasmid transfer. Furthermore, transcipients acquiring only these small B. thuringiensis plasmids (Fig. 1, lane 8) were ineffective in transferring pBC16. In contrast, transcipients inheriting only pBC16 and either pXO11 or pXO12 (Fig. 2, lanes 7 through 10) were capable of transferring pBC16 (Table 2).

B. anthracis and B. cereus transcipients that harbored pXO12 were more fertile than the original donor, B. thuringiensis 4042A UM8 td2, with respect to transfer of pBC16. On the other hand, B. anthracis and B. cereus transcipients that inherited pXO11 were less effective donors of pBC16 than either of the two B. thuringiensis donors, 4042A UM8 td2 and 4042A UM8-13 td1-A. This latter observation suggests that other factors in B. thuringiensis may contribute to the donor activity of pXO11.

Time and frequency of pBC16 transfer by B. anthracis. Fig. 3 shows that the numbers of transcipients from a mating between a B. anthracis donor carrying pXO12 and a B. anthracis recipient increased rapidly during the period between 2 and 6 hours and very slowly after that. The greatest relative increase (164-fold) in transcipients occurred between 2 and 4 hours of mating indicating that many independent transfer events occurred during that period. Although not included here, comparable results were obtained with B. anthracis donors carrying pXO11 and with B. cereus and B. thuringiensis donors carrying either pXO11 or pXO12. In experiments in which mating mixtures were sampled at 0, 30, 60, 90, and 120 min, no transcipients could be detected before 120 min, suggesting that a period of growth of donor and recipient cells together was required prior to plasmid transfer. The necessity for exponential growth of donor and recipient cells together was further supported by the failure to detect transcipients from mating mixtures prepared from donors and recipients which had been grown separately for increasing periods of time (4 to 16 hours) before they were mixed.

Mechanism of transfer. To investigate the possibility of phage-mediated plasmid transfer, we tested cell-free filtrates prepared from donor cultures for the ability to convert recipients to tetracycline-resistance. No Tc^r transcipients could be detected in such experiments. To determine whether

plasmid transfer occurred by transformation, we examined the sensitivity of pBC16 transfer to DNase as described under Materials and Methods. In matings between B. anthracis donors and B. cereus recipients the number of Tc^r transcipients obtained after 4 hours in the presence of DNase and $MgSO_4$ (3.2×10^6 per ml) was not significantly different from the number obtained in the presence of $MgSO_4$ alone (3.0×10^6 per ml). Finally, to determine whether cell-to-cell contact was necessary for plasmid transfer we conducted a mating between a B. anthracis donor and a B. cereus recipient in a U-tube. A 0.45-μm filter (Millipore, type HA) inserted between the two strains prevented cell-to-cell contact but allowed diffusion of filterable material between the two cultures. As a control, the two cultures were also mixed together in a second U-tube without a filter. After 3 hours of incubation normal numbers of transcipients were recovered from the tube without the filter, but no transcipients were detected in samples from the tube containing the filter.

Discussion. The results presented here demonstrate that each of two plasmids, pXO11 and pXO12, indigenous to strain 4042A of B. thuringiensis subsp. thuringiensis, is capable of promoting plasmid transfer within and among strains of B. anthracis, B. cereus, and B. thuringiensis. All transfer proficient B. anthracis and B. cereus transcipients thus far examined inherited either pXO11 or pXO12. That pXO11 and pXO12 can function independently of the small molecular weight plasmids of B. thuringiensis is direct proof that they are fertility plasmids capable of bringing about their own transfer as well as that of other plasmids. Transcipients harboring pXO12 were more effective donors than those harboring pXO11, and B. cereus was generally a better recipient than B. anthracis. For example, maximum frequencies for pBC16 transfer by $(pXO12)^+$ B. anthracis donors to B. anthracis and B. cereus recipients were 5% and 80%, respectively.

Plasmid pXO12 was inherited less frequently than pXO11 by recipients mated with the B. thuringiensis donor which carried both pXO11 and pXO12. However, once the two plasmids were segregated, transcipients inheriting pXO12 were more fertile than those inheriting pXO11. These observations, together with the failure to detect transcipients that acquired both pXO11 and pXO12, suggests that these two fertility plasmids may compete in the transfer process. An analogous competition phenomenon has been reported by Clewell (2) for streptococcal matings in which the transfer ability of the conjugative erythromycin-resistance plasmid, pAMβ1, is drastically reduced in the presence

of either of two other conjugative plasmids, pAMα1 and pAD1. Incompatibility is a universal trait exhibited among plasmids which are isogenic for maintenance, replication, and transfer functions, and this may explain the apparent competition between pXO11 and pXO12 and the spontaneous loss of pXO12 from B. thuringiensis 4042A UM8-13 td1-A.

Although the mechanism of transfer is still unknown, several lines of evidence support a conjugation-like process: (1) the addition of DNase to mating mixtures did not reduce transfer frequencies; (2) donor filtrates were inactive and cell-to-cell contact was necessary; (3) the high frequencies of transfer are typical of conjugation systems; (4) the large increase in numbers of transcipients between 2 and 4 hours (10^2- to 10^5-fold) indicates that many independent transfer events occurred. Our results showing a requirement for cell-to-cell contact and the ineffectiveness of DNase in preventing plasmid transfer from B. anthracis donors are similar to those obtained by Gonzalez and Carlton (6) in plasmid transfer experiments with B. thuringiensis donors.

There appeared to be an essential period (2 to 4 hours) of growth of donor and recipient cells together before plasmid transfer could be detected. The requirement for growing donor and recipient cells together during the exponential phase of growth was dramatically illustrated by the drastic reduction in plasmid transfer when mating mixtures were prepared from donor and recipient cells grown separately for similar periods of time (data not shown).

In addition to transfer functions, the fertility plasmid, pXO12, was found to carry information involved in parasporal crystal formation which was expressed in all three species of Bacillus tested. The evidence for this was two-fold: (1) All transcipients harboring pXO12 were Cry^+ while those harboring pXO11 were Cry^-, and (2) the inability of strain 4042A UM8-13 td1-A to produce parasporal crystals when infected with the converting phage TP-13 (see footnote, Table 1) correlated with the spontaneous loss of pXO12.

Future work to determine the extent of homology between pXO11 and pXO12 may provide further insight into their apparent incompatibility and their maintenance and transfer functions. Presently, the ability to transfer a large range of plasmids makes this a useful genetic exchange system for the functional analysis of genetic determinants on plasmids of B. anthracis, B. cereus, and B. thuringiensis.

IV. Studies with the B. anthracis toxin plasmid, pXO1

Size determination of pXO1 by restriction analysis. Purified pXO1 DNA, isolated from the Weybridge strain of B. anthracis, was fragmented with HindIII or EcoRI. The mobilities of the fragments during electrophoresis in agarose gels were compared with those of standard fragments (lambda DNA fragmented with the same enzymes and also gel marker DNA obtained from Bethesda Research Laboratories). Electrophoresis was carried out in 0.5% and 1.2% agarose gels. Standard curves comparing mobility with known fragment size were constructed for gel marker DNA and lambda DNA fragments. Large pXO1 fragments were assigned size values from the curve constructed with data from 0.5% gels and smaller pXO1 fragments were assigned size values from the curve constructed with data from 1.2% gels. The size of pXO1 estimated from the HindIII digest was 171.5 kilobase pairs and the size estimated from the EcoRI digest was 177.0 kilobase pairs. The average value from the two determinations, 174.3 kilobase pairs, is in good agreement with the value of 168.4 plus or minus 7.3 kilobase pairs as estimated by Vodkin and Leppla (25) from electron microsopic measurements. Complete details of these analyses were given by Robillard (18).

Hybridization studies with pXO1 as a probe. To determine whether pXO1 had integrated into the bacterial chromosome of strains presumed to be cured of the plasmid and also to explore the possibility that pXO1 and the chromosome might have sequences in common, we carried out experiments using the Southern hybridization technique. ^{32}P-nick translated pXO1 DNA was used to probe whole DNA from the parent Weybridge strain and from three derivatives cured of pXO1 by three different methods. DNA extracted from the parent strain readily hybridized to ^{32}P-labeled pXO1 DNA. However, DNA from the cured strains failed to hybridize with the labeled plasmid DNA to a detectable extent. These results suggest very strongly that pXO1 is completely absent from the cured strains and also that pXO1 does not share homologous sequences with the chromosome. Further details of these experiments can be found in Robillard's Ph. D. dissertation (18).

Transfer of pXO1 to B. cereus and cured strains of B. anthracis by mating. We have succeeded in demonstrating the transfer of pXO1 from cell to cell during the mating process mediated by the B. thuringiensis plasmid, pXO12. When B.

anthracis Weybridge A UM2 tr244-1, a transcipient carrying the three plasmids, pXO1, pXO12, and pBC16, was mated with a $(pXO1)^-$ strain, UM44-1-C9 Ind^-Str^r, two Tc^rStr^r transcipients out of 475 tested on halo agar for zones of precipitation were positive (PA^+). These two transcipients, labeled UM44-1-C9 tr41G-1 and tr41G-2, were both shown to carry the three plasmids, pXO1, pXO12, and pBC16. They were confirmed to produce protective antigen in broth culture.

From a mating experiment in which UM44-1-C9 tr41G-1 was the donor and Weybridge A UM23C1-1 $Ura^-Str^r(pXO1)^-$ was the recipient, three out of 64 Tc^r transcipients tested on halo agar for protective antigen synthesis were positive. The three transcipients were labeled Weybridge A UM23C1-1 tr47G-6, -34, and -61. The first two were also Cry^+ and the third one was Cry^-. Plasmid analysis on agarose gels revealed that lysates of each of the three transcipients had only one plasmid band which migrated more slowly than chromosomal DNA, and it was apparently larger than pXO1 or pXO12. This is in contrast to UM2 tr244-1 and UM44-1-C9 tr41G-1 in which both pXO1 and pXO12 could be demonstrated. Thus, it appeared that the newly observed high molecular weight band found in each of the three transcipients, tr47G-6, -34, and -61, represented a cointegrate or recombinant plasmid.

These three transcipients were tested for the ability to transfer pBC16 when mated with B. cereus 569 UM20-1. The results are shown in Table 3. As expected, only the two transcipients that were Cry^+ were able to mobilize pBC16. The numbers of Tc^r transcipients obtained are typical of those observed from matings of Cry^+Tc^r B. anthracis donors with B. cereus recipients.

Sixty-two B. cereus transcipients from the mating in which UM23C1-1 tr47G-34 was the donor (Table 3) were tested on halo agar for protective antigen production, and at least 35 formed a zone of precipitation. (Because B. cereus is motile, the colonies spread considerably on halo agar and it is sometimes difficult to determine whether there is a zone of precipitation.) A number of PA^+ and PA^- transcipients were examined for plasmid content, and the results are confusing. A variety of large plasmids were found among the transcipients. The majority of PA^+ transcipients had two plasmids above chromosomal DNA, and they appeared to correspond to pXO1 and pXO12. A minority of the PA^+ transcipients had only one band migrating above chromosomal DNA and it resembled that present in the donors. Those transcipients that were PA^- had either no plasmid DNA bands above chromosomal DNA or one that migrated to the position of pXO12.

These results suggest that pXO1 and pXO12 can form a cointegrate and/or

recombinant plasmid, such as that found in B. anthracis UM23C1-1 tr47G-34, and that it is sometimes resolved during or after transfer. The much higher frequency of PA^+ transcipients found in matings with the "cointegrate" donor, compared to the frequencies found in matings with donors carrying resolved pXO1 and pXO12, suggests that the resolution may occur after transfer. The fact that transcipient UM23C1-1 tr47G-61, which also apparently contained a cointegrate or recombinant plasmid, was Cry^- and nonfertile (Table 3), suggests that sequences involved in the synthesis of parasporal crystal and in fertility were lost or rearranged during formation of the complex plasmid.

These results are very recent and we have not answered the many questions regarding the complex array of plasmids we are seeing in transcipients. For that reason we have not included photographs of agarose gels showing migration of the various plasmids. More precise information regarding the nature of the presumed cointegrates must await restriction analyses of the plasmids involved. In the meantime we plan to test PA^+ B. cereus transcipients for protective antigen production in broth culture.

Comparison of characteristics of cured and uncured strains of B. anthracis. As reported previously (Annual Report, December 1982 and December 1983), a number of cured derivatives $(pXO1)^-$ have been isolated from several genetically marked mutants of the Weybridge strain. In addition to the loss of ability to produce protective antigen, all cured strains we have tested are similar with respect to the following characteristics: (1) Cured cells sporulate earlier and at a higher frequency than uncured cells; (2) Colonies of cured cells differ from colonies of uncured cells in their morphology; (3) Cured cells are more sensitive than uncured cells to certain bacteriophages; (4) Cured cells do not grow as well as uncured cells in certain synthetic media; and (5) Spore-negative mutants are found at a much higher frequency in cultures of cured strains than in cultures of $(pXO1)^+$ strains.

Proof that the altered characteristics listed above were, in fact, a result of plasmid loss rather than a consequence of selection procedures used in isolating cured strains could be established only by transferring pXO1 back into strains previously cured of the plasmid. This was made possible by development of the mating system discussed above. Cured strains that were reinfected with pXO1 by the mating process could not be distinguished from the uncured parent strain from which they were originally derived. This confirms conclusively that the phenotypic changes listed above, including loss of ability to produce

protective antigen, are consequences of plasmid loss.

V. Studies with the B. anthracis capsule plasmid, pXO2

Noncapsulated (rough) variants of the Pasteur strain, ATCC 6602. When Cap^+ strains of B. anthracis are grown under conditions conducive to capsule formation, i.e., on agar containing bicarbonate and/or serum and incubated in 20% CO_2, rough sectors or edges occur frequently among the mucoi. colonies. Once it was found that strain 6602 carries the plasmid, pXO2, which is associated with capsule formation, it became of interest to investigate rough variants of this strain with respect to plasmid content. Therefore, we isolated a number of noncapsulated rough variants from rough sectors or edges of mucoid colonies grown on NBY agar containing 0.7% sodium bicarbonate and 10% horse serum and incubated in approximately 20% CO_2.

Plasmid analyses revealed that there were two classes of rought variants. Some of the rough variants had been cured of pXO2 and contained no detectable plasmids, but other rough variants still carried pXO2. We then predicted that those rough variants which retained pXO2 could be demonstrated to revert to Cap^+, but that reversion to Cap^+ could not be demonstrated in those which had been cured of the plasmid. This prediction was found to be true. Both classes of rough variants were tested for reversion by exposing them to the virulent bacteriophage, Wα, which lyses rough cells but not those that are capsulated. No mucoid revertants were found among three independently isolated $(pXO2)^-$ strains; however, mucoid revertants were found in each of three independently isolated rough strains that retained pXO2. Presumably, rough variants that carry pXO2 are rough because they carry point mutations. This observation explains the reports by Thorne (22) and Meynell (13) that some noncapsulated variants of B. anthracis were stable and others reverted to Cap^+. Plasmid profiles of the Pasteur strain 6602 and a rough Cap^- $(pXO2)^-$ variant are shown in Fig. 4 (lanes 1 and 2).

In view of the above demonstration that revertible rough variants still carry pXO2, it becomes of interest to determine whether the mutations that engender the rough phenotype are chromosomal or plasmid borne. It seems possible that such mutations could reside on either replicon. Now that we are able to transfer pXO2 by the mating system we are in a position to test whether a particular rough variant carries a mutation on the chromosome or on the

plasmid. If the mutation is chromosomal, presumably transcipients that inherit pXO2 from the rough variant would be Cap^+.

Transfer of pXO2 to B. cereus and its expression in that host. Proof that plasmid pXO2 confers on its host the ability to produce capsules was obtained by transferring the plasmid to B. cereus 569. To do this we first put the fertility plasmid, pXO12, into strain 6602 by mating the donor, B. anthracis Weybridge A UM23C2 tr60B-1 Ura^- $(pXO1)^-(pXO12)^+(pBC16)^+$, with strain 6602 and selecting transcipients that were Tc^r and Cry^+, i.e., $(pBC16)^+$ and $(pXO12)^+$, on Min 1C agar (counterselecting the Ura^- donor). Such 6602 transcipients were shown to carry the three plasmids, pXO2, pBC16, and pXO12. One of these transcipients, 6602 tr172B-2 (Fig. 4, lane 3), was mated with B. cereus 569 UM20-1 Ant^-Str^r, and Tc^r Str^r transcipients were selected on NBY agar containing bicarbonate and serum plus tetracycline and streptomycin and incubated in 20% CO_2. The frequency of Tc^r transcipients was normal, i.e., about 6×10^5 per ml, but only one in 500 of these was mucoid. One of these, B. cereus 569 UM20-1 tr49G-4 (Fig. 4, lane 5), was chosen for further study. It was Cap^+ when grown in CO_2, produced crystals when it was allowed to sporulate, and retained the Ant^- and Str^r markers of the original recipient. Capsule production by a B. cereus transcipient and comparison of the transcipient with the Pasteur strain, ATCC 6602, are demonstrated in the photographs of Figures 5, 6, 7, and 8.

The two plasmids, pXO2 and pXO12, separated poorly if at all when lysates of the Cap^+Cry^+ B. cereus transcipient, 569 UM20-1 tr49G-4, were subjected to electrophoresis in agarose gels (Fig.4, lane 5). This is discussed below.

Further studies on transfer of the capsule plasmid, pXO2. For further confirmation of the mobilization of pXO2 the mating described above was repeated, i.e., B. anthracis 6602 tr172B-2 was the donor and B. cereus 569 UM20-1 Ant^-Str^r was the recipient. Tc^rStr^r transcipients were selected on NBY agar supplemented with tetracycline, streptomycin, bicarbonate, and serum and incubated in 20% CO_2. Five Cap^+ transcipients were isolated and found to produce capsules under the same conditions required for capsule formation by B. anthracis strain 6602. All five retained the Ant^- marker of the B. cereus recipient, and all five were Cry^+. The plasmid content of the five Cap^+Cry^+ transcipients and three Cap^-Cry^+ transcipients was examined. The plasmid profiles of four of the five Cap^+ transcipients were identical to the profile of the donor, 569 UM20-1 tr49G-4(Fig. 9, lane 8), i.e., above the chromosomal DNA there was a single band which migrated to the same position as pXO12 (tr60G-6 in

Fig. 9, lane 3). Each of the three Cap^- transcipients also had only one band above the chromosome (tr60G-11 in Fig. 9, lane 5), and it migrated to the position of pXO12 but was less intense than the corresponding band in the Cap^+ transcipients. The fifth Cap^+ transcipient to be analyzed (569 UM20-1 tr60G-10) was different, however, in that no band was present where pXO12 migrates, but instead, a plasmid larger than either pXO2 or pXO12 was present (Fig. 9, lane 4). Presumably it is a cointegrate of pXO2 and pXO12 since tne transcipient is both Cap^+ and Cry^+.

If the large plasmid found in tr60G-10 is a cointegrate of pXO2 and pXO12, we hypothesized that if its host were used as a donor in the mating system, all transcipients that acquired the plasmid would be both Cap^+ and Cry^+. Therefore, a mating was carried out in which 569 UM20-1 tr60G-10 was the donor and B. anthracis Weybridge A UM23C1-2 $Ura^-Rif^r(pXO1)^-$ was the recipient. Tc^rRif^r transcipients were selected on NBY agar supplemented with rifampicin, tetracycline, bicarbonate, and serum and incubated in 20% CO_2. Approximately 1 x 10^4 transcipents per ml were found after 6 hours of mating, and 12% of them were Cap^+. We examined 17 Cap^+ and 38 Cap^- transcipients for parasporal crystal production and, as expected, all transcipients which were Cap^+, and only those which were Cap^+, produced crystals. Thus far, only one Cap^- and three Cap^+ transcipients have been examined for plasmid content. All three Cap^+Cry^+ transcipients contained a plasmid (Fig. 9, lane 7) the same size as the suspected cointegrate plasmid in the donor (Fig. 9, lane 6), and the Cap^- transcipient contained no plasmid that migrated above the chromosomal DNA (not shown). These observations are consistent with the interpretation that the large plasmid found in Cap^+Cry^+ transcipients is a cointegrate or recombinant plasmid formed between pXO2 and pXO12.

A question still remaining is why both pXO2 and pXO12 can not be visualized in electrophoretic gels of Cry^+Cap^+ B. cereus transcipients such as 569 UM20-1 tr49G-4 discussed above. Let it be recalled that such transcipients appear to contain only one large plasmid migrating slower than chromosomal DNA and it migrates at the same rate as pXO12. Because both pXO2 and pXO12 can be seen in B. anthracis 6602 tr172B-2, which was the original donor for 569 UM20-1 tr49G-4 (Fig. 9, lane 8), we thought that if 569 UM20-1 tr49G-4 were used as the donor in a mating with B. anthracis recipients, the two plasmids might be resolved in the transcipients. The mating was carried out with B. anthracis UM23C1-2 $Ura^-Rif^r(pXO1)^-$ as the recipient, and Tc^rRif^r transcipients were selected on NBY

agar supplemented with rifampicin, tetracycline, bicarbonate, and serum and incubated in 20% CO_2. Nine Cap^+ Cry^+ and four Cap^-Cry^+ transcipients were examined for their plasmid content. In no instance was there evidence of both pXO2 and pXO12; all contained only one plasmid band above the chromosomal DNA and it migrated to the same position as pXO12. Again, however, when the transcipients were Cap^+Cry^+ (Fig.9, lane 9), the plasmid band was more intense than the corresponding band in transcipients that were Cap^-Cry^+ (Fig. 9, lane 10). One possible explanation is that the plasmid in Cap^+Cry^+ transcipients which gives a more intense band than the plasmid band given by Cap^-Cry^+ transcipients is a recombinant plasmid made up mostly of pXO12 but carrying capsule genes from pXO2. Hopefully, we will be able to resolve some of the questions concerning these presumed cointegrate and recombinant plasmids by restriction analyses of the plasmids involved.

TABLE 1. Strains used in this study

Strain	Relevant Characteristics[a]	Relevant Plasmids[b]	Origin/reference
B. anthracis			
M strain	Cap^+ in air	pXO1, pXO2	Origin unknown[c]
Pasteur 6602	Avirulent, Cap^+Tox^-	pXO2	ATCC[d] 6602
Pasteur 6602 R1	Avirulent, Cap^-	None	This study
Pasteur 6602 tr172B-2	Avirulent, $Cap^+Cry^+Tc^r$	pXO2, pXO12, pBC16	This study
Weybridge	Avirulent, Cap^-Tox^+	pXO1[b]	MRE[e]
Weybridge A	Colonial variant of Weybridge	pXO1	C. B. Thorne
Weybridge A UM2	Ind^-	pXO1	UV[f] of Weybridge A
Weybridge A UM2 tr244-1	$Ind^-Tc^rCry^+$	pXO1, pXO12, pBC16	This study
Weybridge A UM17	Ade^-	pXO1	UV of Weybridge A
Weybridge A UM17 tr57B-6	$Ade^-Tc^rCry^+$	pXO1, pXO12, pBC16	This study
Weybridge A UM23	Ura^-	pXO1	UV of Weybridge A
Weybridge A UM23C1	Ura^-, Cured of pXO1	None	C. B. Thorne
Weybridge A UM23C1-1	Ura^-Str^r	None	UV of A UM23C1
Weybridge A UM23C1-1 tr47G-6	$Ura^-Tc^rCry^+PA^+$	See text	This study

TABLE 1. (continued)

Weybridge A UM23C1-1 tr47G-34	$Ura^-Tc^rCry^+PA^+$	See text	This study
Weybridge A UM23C1-1 tr47G-61	$Ura^-Tc^rCry^-PA^+$	See text	This study
Weybridge A UM23C1-2	Ura^-Rif^r	None	UV of A UM23C1
Weybridge A UM23C1-2 tr63G-10	$Ura^-Tc^rRif^rCap^+Cry^+$	See text	This study
Weybridge A UM23C1-2 tr63G-21	$Ura^-Tc^rRif^rCap^-Cry^+$	See text	This study
Weybridge A UM23C1-2 tr66G-1	$Ura^-Tc^rRif^rCap^+Cry^+$	See text	This study
Weybridge A UM23C2	Ura^-, cured of pXO1	None	C. B. Thorne
Weybridge A UM23C2 tr45B-12	$Ura^-Tc^rCry^-$	pXO11, pBC16	This study
Weybridge A UM23C2 tr60B-1	$Ura^-Tc^rCry^+$	pXO12, pBC16	This study
Weybridge A UM23C2 tr96B-3	$Ura^-Tc^rCry^-$	pXO11, pBC16	This study
Weybridge A UM23C2 tr237-10	$Ura^-Tc^rCry^+$	pXO12, pBC16	This study
Weybridge UM44	Ind^-	pXO1	UV of Weybridge
Weybridge UM44-1	Ind^-Str^r	pXO1	UV of UM44
Weybridge UM44-1 tr84-6	$Ind^-Str^rTc^rCry^-$	pXO1, pXO11, pBC16	This study
Weybridge UM44-1 tr84-7	$Ind^-Str^rTc^rCry^-$	pXO1, pXO11, pBC16	This study
Weybridge UM44-1 tr203-1	$Ind^-Str^rTc^rCry^+$	pXO1, pXO12, pBC16	This study
Weybridge UM44-1 tr203-7	$Ind^-Str^rTc^rCry^+$	pXO1, pXO12, pBC16	This study

TABLE 1 (continued)

Weybridge UM44-1 tr203-23	$Ind^-Str^rTc^rCry^+$	pXO1, pXO12, pBC16	This study
Weybridge UM44-1 tr203-28	$Ind^-Str^rTc^rCry^-$	pXO1, pXO11, pBC16	This study
Weybridge UM44-1-C9	Ind^-Str^r, Cured of pXO1	None	This study
Weybridge UM44-1-C9 tr41G-1	$Ind^-Tc^rStr^rCry^+PA^+$	pXO1, pXO12, pBC16	This study
Weybridge UM44-1-C9 tr41G-2	$Ind^-Tc^rStr^rCry^+PA^+$	pXO1, pXO12, pBC16	This study
B. cereus			
569	wild type		NRRL[g]
569 UM20	Ant^-		UV of 569
569 UM20-1	Ant^-Str^r	One unnamed	UV of UM20
569 UM20-1 tr2B-1	$Ant^-Str^rTc^rCry^-$	pXO11, pBC16	This study
569 UM20-1 tr2B-3	$Ant^-Str^rTc^rCry^-$	pXO11, pBC16	This study
569 UM20-1 tr2B-4	$Ant^-Str^rTc^rCry^-$	pXO11, pBC16	This study
569 UM20-1 tr49G-4	$Ant^-Str^rTc^rCry^+Cap^+$	See text	This study
569 UM20-1 tr60G-6	$Ant^-Str^rTc^rCap^+Cry^+$	See text	This study
569 UM20-1 tr60G-10	$Ant^-Str^rTc^rCap^+Cry^+$	See text	This study
569 UM20-1 tr60G-11	$Ant^-Str^rTc^rCap^-Cry^+$	See text	This study
569 UM20-1 tr195B-35	$Ant^-Str^rTc^rCry^+$	pXO12, pBC16	This study

TABLE 1 (continued)

569 UM20-1 tr210B-1	$Ant^{-}Str^{r}Tc^{r}Cry^{-}$	pXO11, pBC16	This study
569 UM20-1 tr251-1	$Ant^{-}Str^{r}Tc^{r}Cry^{+}$	pXO12, pBC16	This study
569 UM20-1 tr251-5	$Ant^{-}Str^{r}Tc^{r}Cry^{-}$	pBC16	This study
11950	Carries bacteriophage Wα		ATCC
B. thuringiensis			
4042A	subsp. thuringiensis	pXO11, pXC12	(1)
4042A UM8	$Ade^{-}Cry^{+}$	pXO11, pXO12	UV of 4042A
4042A UM8 $td2^{h}$	$Ade^{-}Cry^{+}Tc^{r}$	pXO11, pXO12, pBC16	C. B. Thorne
4042A UM8-13	$Ade^{-}Cry^{-}Osp$	pXO11, pXO12	C. B. Thorne[i]
4042A UM8-13 td1	$Ade^{-}Cry^{-}Osp\ Tc^{r}$	pXO11, pXO12, pBC16	C. B. Thorne[i]
4042A UM8-13 td1-A	$Ade^{-}Cry^{-}Osp\ Tc^{r}$	pXO11, $(pXO12)^{-}$, pBC16	C. B. Thorne[i]

[a]Abbreviations: Ade, adenine; Ant, anthranilic acid; Cap, synthesis of capsule; Cry, synthesis of parasporal crystals; Ind, indole; Osp, oligosporogenous; PA, synthesis of protective antigen; Rif^{r}, rifampicin-resistant; Str^{r}, streptomycin-resistant; Tc^{r}, pBC16-encoded tetracycline resistance; Tox, synthesis of toxin; tr, transcipient; Ura, uracil.

[b]pXO1 encodes synthesis of anthrax toxin; pXO2 carries genes involved in capsule synthesis; pXO11 is a fertility plasmid from B. thuringiensis; pXO12 is a fertility plasmid from B. thuringiensis and it encodes parasporal crystal formation; pBC16 encodes resistance to tetracycline.

TABLE 1 (concluded)

[c]The origin of the M strain is unknown. It is a mutant which produces capsules when grown in air in the absence of serum or added bicarbonate.

[d]ATCC, American Type Culture Collection.

[e]MRE, Microbiological Research Establishment, Porton, England.

[f]UV, Mutagenesis by UV light.

[g]NRRL, Agricultural Research Service, Northern Regional Research Laboratory, U.S. Department of Agriculture, Peoria, Ill.

[h]td, Tc^r transductant obtained by phage CP-51-mediated transfer of pBC16.

[i]Although 4042A UM8-13 was Osp and Cry^-, it contained pXO12 and could be converted to Spo^+Cry^+ by phage TP-13 (14). UM8-13 td1, a Tc^r transductant of UM8-13, could also be converted to Spo^+Cry^+. Following frequent transfers of UM8-13 td1 on L-agar slants it was found to have lost pXO12 and, consequently, could not be converted to Cry^+ by TP-13. The $(pXO12)^-$ derivative is designated UM8-13 td1-A. Freeze dried preparations of UM8-13 td1 retained pXO12.

TABLE 2. Effectiveness of B. anthracis and B. cereus transcipients as donors in the transfer of pBC16[a]

Donor	Tc^r transcipients when mated with			
	B. anthracis		B. cereus	
	No. per ml	Frequency	No. per ml	Frequency
Primary Transcipients				
B. anthracis Weybridge				
UM44-1 tr84-6(pXO1,pXO11,pBC16) Cry$^-$	4.9×10^2	1.1×10^{-5}	1.5×10^2	3.5×10^{-6}
UM44-1 tr203-1(pXO1,pXO12,pBC16) Cry$^+$	2.7×10^5	6.3×10^{-3}	1.0×10^7	2.3×10^{-1}
UM44-1 tr203-7(pXO1,pXO12,pBC16) Cry$^+$	1.3×10^5	3.0×10^{-3}	7.5×10^6	1.7×10^{-1}
UM44-1 tr203-23(pXO1,pXO12,pBC16) Cry$^+$	1.2×10^5	2.8×10^{-3}	2.6×10^7	6.0×10^{-1}
UM44-1 tr203-28(pXO1,pXO11,pBC16) Cry$^-$	5.0×10^2	1.2×10^{-5}	4.2×10^2	9.8×10^{-6}
B. cereus 569				
UM20-1 tr2B-1(pXO11,pBC16) Cry$^-$	3.5×10^1	8.1×10^{-7}		
UM20-1 tr2B-3(pXO11,pBC16) Cry$^-$	4.5×10^1	1.0×10^{-6}		
UM20-1 tr2B-4(pXO11,pBC16) Cry$^-$	2.0×10^1	4.7×10^{-7}		

Table 2 (continued)

Secondary Transcipients				
B. anthracis Weybridge				
UM17 tr57B-6(pXO1, pXO12, pBC16) Cry^+	1.0×10^5	2.3×10^{-3}	2.1×10^6	4.9×10^{-2}
UM23C2 tr45B-12(pXO11, pBC16) $(pXO1)^-$ Cry^-	6.7×10^2	1.6×10^{-5}	2.7×10^2	6.3×10^{-6}
UM23C2 tr60B-1(pXO12, pBC16) $(pXO1)^-$ Cry^+	8.0×10^4	1.9×10^{-3}	2.0×10^6	4.7×10^{-2}
UM23C2 tr96B-3(pXO11, pBC16) $(pXO1)^-$ Cry^-	8.8×10^2	2.0×10^{-5}	5.2×10^2	1.2×10^{-5}
UM23C2 tr237-10(pXO12, pBC16) $(pXO1)^-$ Cry^+	7.9×10^5	1.8×10^{-2}	3.5×10^7	8.1×10^{-1}
B. cereus 569				
UM20-1 tr210B-1(pXO11,pBC16) Cry^-	7.1×10^1	1.6×10^{-7}	3.5×10^1	8.0×10^{-8}
UM20-1 tr251-1(pXO12,pBC16) Cry^+	1.3×10^5	3.0×10^{-4}	8.6×10^5	2.0×10^{-3}
UM20-1 tr251-5(pBC16) Cry^-	0	0	0	0

[a]To permit selection and identification of Tc^r transcipients we used *B. cereus* and *B. anthracis* recipients which had auxotrophic requirements different from those of the respective donors. Mating mixtures were incubated 20 hours. The average number of *B. anthracis* donors per ml was 4.3×10^7 CFU and that of *B. cereus* donors was 4.4×10^8 CFU.

TABLE 3. High frequency transfer to B. cereus of a presumed cointegrate plasmid carrying information for protective antigen synthesis[a]

Mating mixture No.	Donor	Tc^r transcipients	
		No. per ml	No. PA^+/No. tested
72G	Weybridge A UM23C1-1 tr47G-6 PA^+Cry^+	1.4×10^6	Not tested
73G	Weybridge A UM23C1-1 tr47G-34 PA^+Cry^+	1.6×10^6	35/62 (56%)
74G	Weybridge A UM23C1-1 tr47G-61 PA^+Cry^-	0	

[a]The recipient was B. cereus 569 UM20-1 Ant^-Str^r

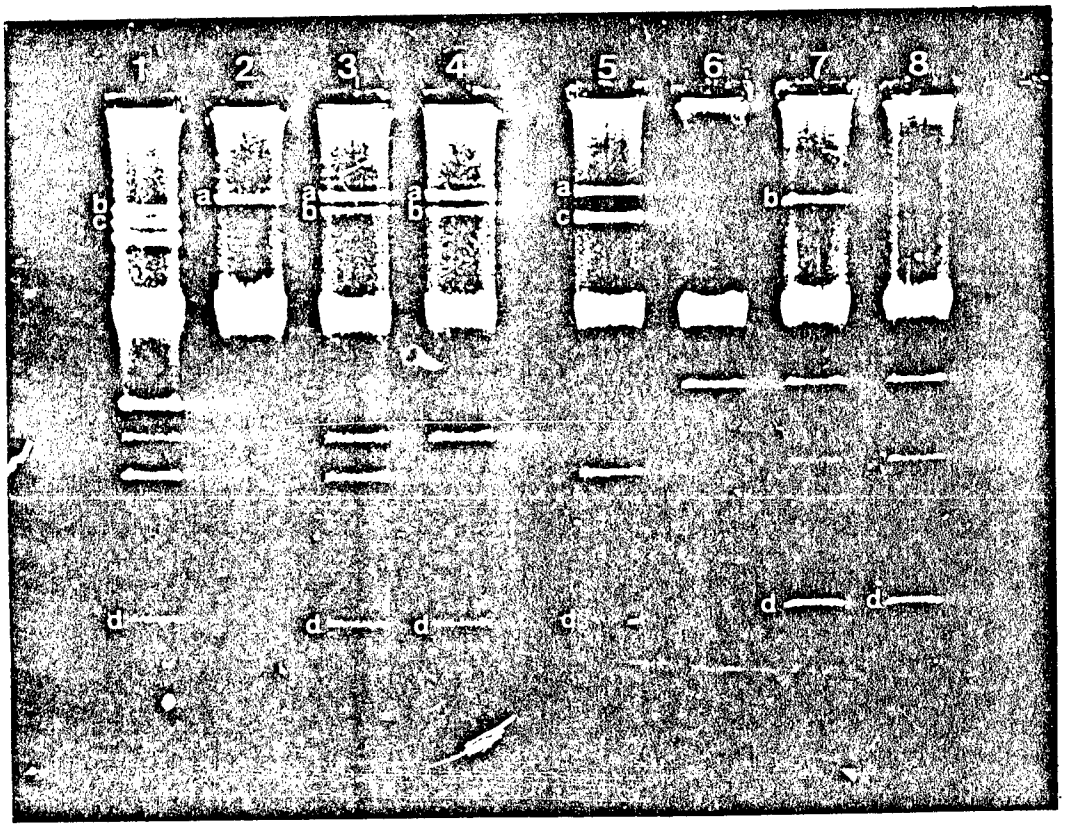


FIG. 1. Agarose gel electrophoresis of plasmid DNA from a B. thuringiensis donor and B. anthracis and B. cereus recipients and transcipients. Plasmids are labeled as follows: (a) pXO1; (b) pXO12; (c) pXO11; (d) pBC16. The large diffuse band in all lanes is chromosomal DNA. Lanes: 1, B. thuringiensis 4042A UM8 td2, Cry^+ donor; 2, B. anthracis Weybridge UM44-1, recipient; 3, Weybridge UM44-1 tr203-1, Cry^+ transcipient; 4, Weybridge UM44-1 tr203-7, Cry^+ transcipient; 5, Weybridge UM44-1 tr203-28, Cry^- transcipient; 6, B. cereus 569 UM20-1, recipient; 7, 569 UM20-1 tr251-1, Cry^+ transcipient; 8, 569 UM20-1 tr251-5, Cry^- transcipient.

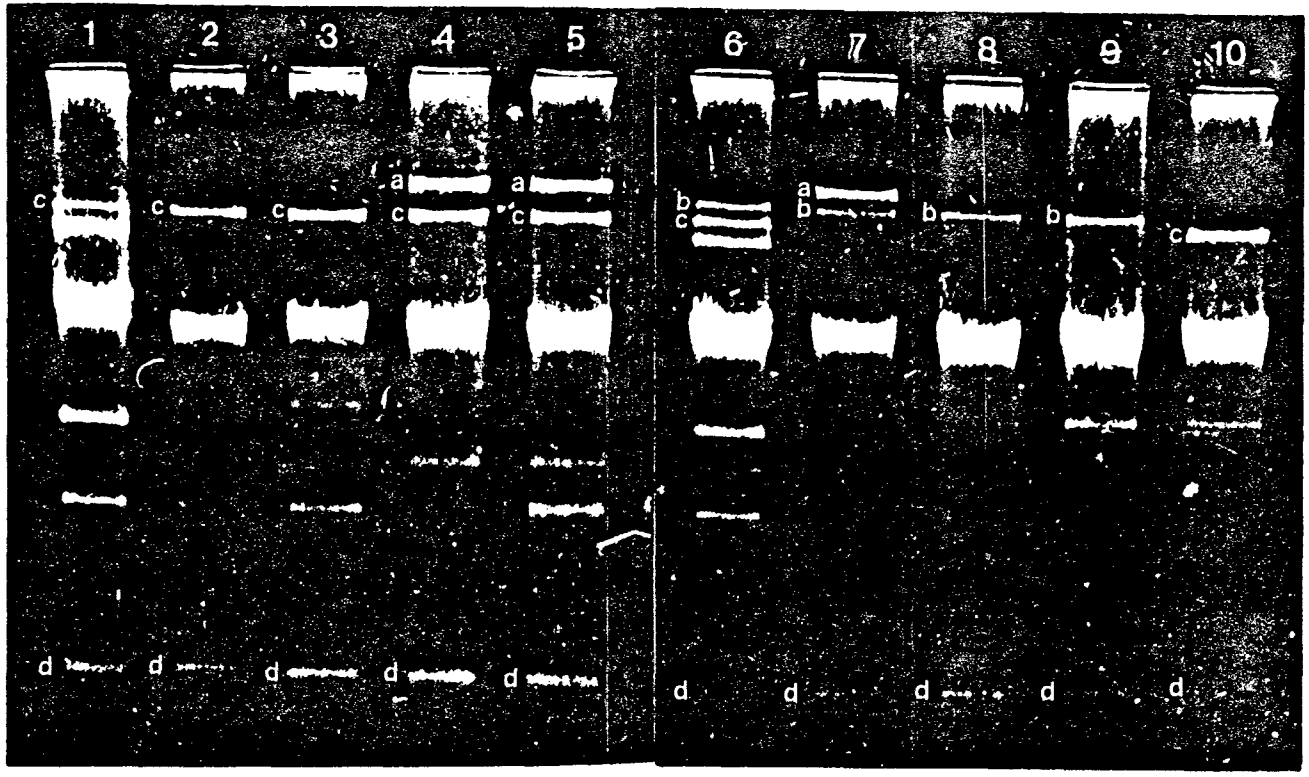


FIG. 2. Agarose gel electrophoresis of plasmid DNA from B. thuringiensis donor strains and B. anthracis and B. cereus transcipients. Plasmid designations are the same as in FIG. 1. Lanes: 1, B. thuringiensis 4042A UM8-13 td1-A, Cry$^-$ donor; 2, B. cereus 569 UM20-1 tr2B-3, Cry$^-$ transcipient; 3, 569 UM20-1 tr2B-1, Cry$^-$ transcipient; 4, B. anthracis Weybridge UM44-1 tr84-6. Cry$^-$ transcipient; 5, Weybridge UM44-1 tr84-7, Cry$^-$ transcipient; 6, B. thuringiensis 4042A M8 td2, Cry$^+$ donor; 7, B. anthracis Weybridge UM44-1 tr203-23, Cry$^+$ transcipient; 8, Weybridge A UM23C2 tr237-10, Cry$^+$ transcipient; 9, B. cereus 569 UM20-1 tr195B-35, Cry$^+$ transcipient; 10, 569 UM20-1 tr2B-4, Cry$^-$ transcipient. The very faint bands below chromosomal DNA are polymeric forms of pBC16.

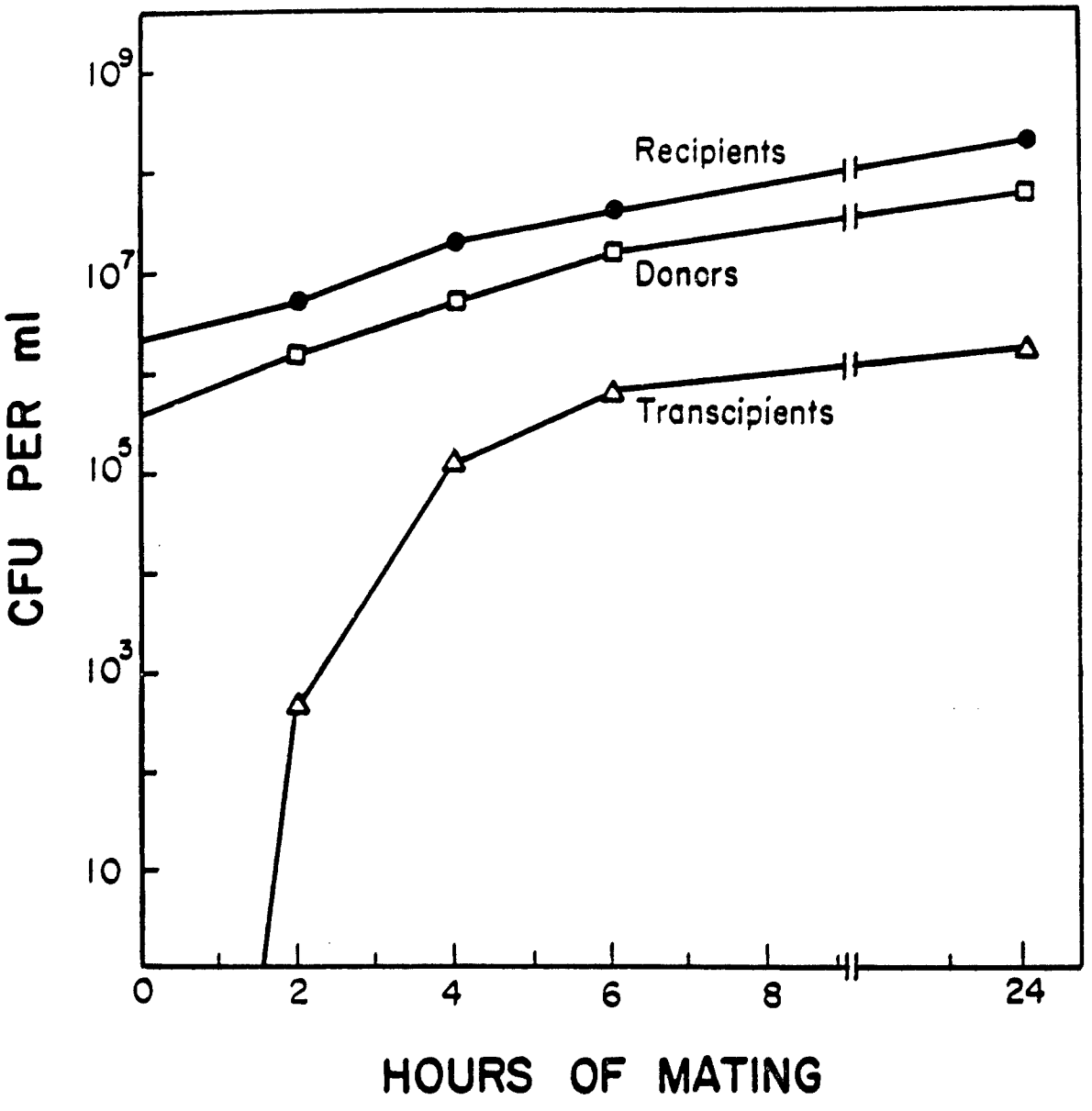


FIG. 3. Transfer of pBC16 from B. anthracis Weybridge A UM23C2 tr237-10(pXO12, pBC16) $Ura^{-}Tc^{r}Cry^{+}$ to B. anthracis Weybridge UM44-1 $Ind^{-}Str^{r}$. At the indicated times samples were plated on L-agar containing tetracycline or streptomycin or both antibiotics to score donors, recipients, and transcipients, respectively.

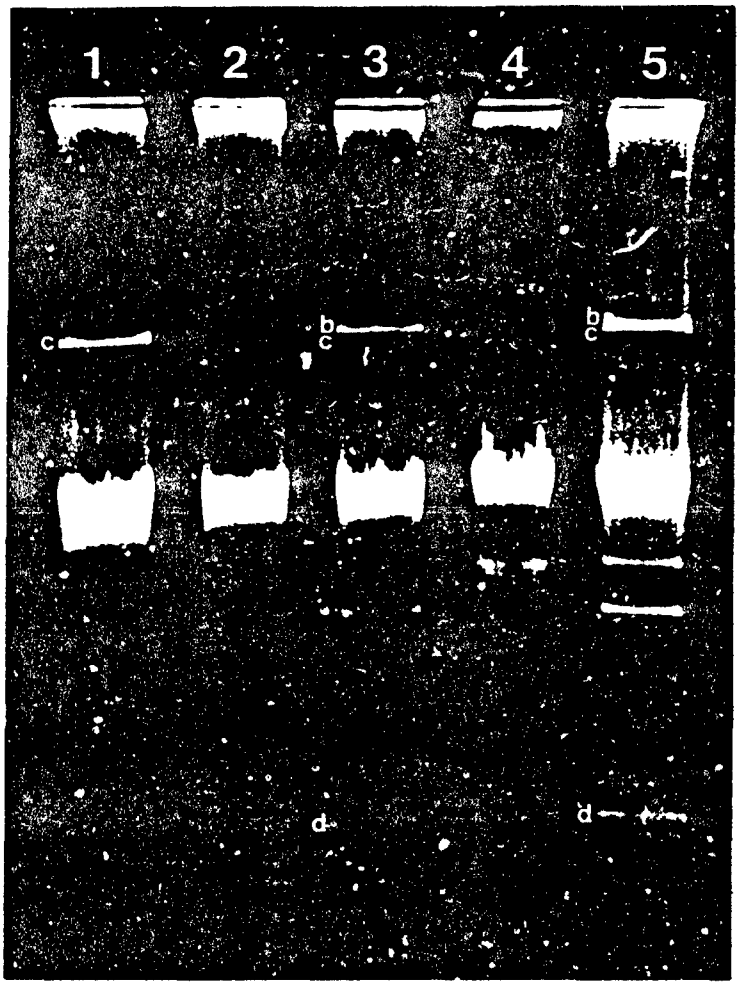


FIG. 4. Agarose gel electrophoresis of plasmid DNA from B. anthracis and B. cereus, demonstrating pXO2 and its transfer to B. cereus. Lane 1, B. anthracis 6602 wild type; Lane 2, B. anthracis 6602 R1 (rough variant); Lane 3, B. anthracis 6602 tr172B-2, a transcipient carrying pXO2, pXO12, and pBC16; Lane 4, B. cereus 569 UM20-1; Lane 5, B. cereus 569 UM20-1 tr49G-4, a transcipient carrying pXO2, pXO12, and pBC16. Plasmids are labeled as follows: (b) pXO12; (c) pXO2; (d) pBC16.

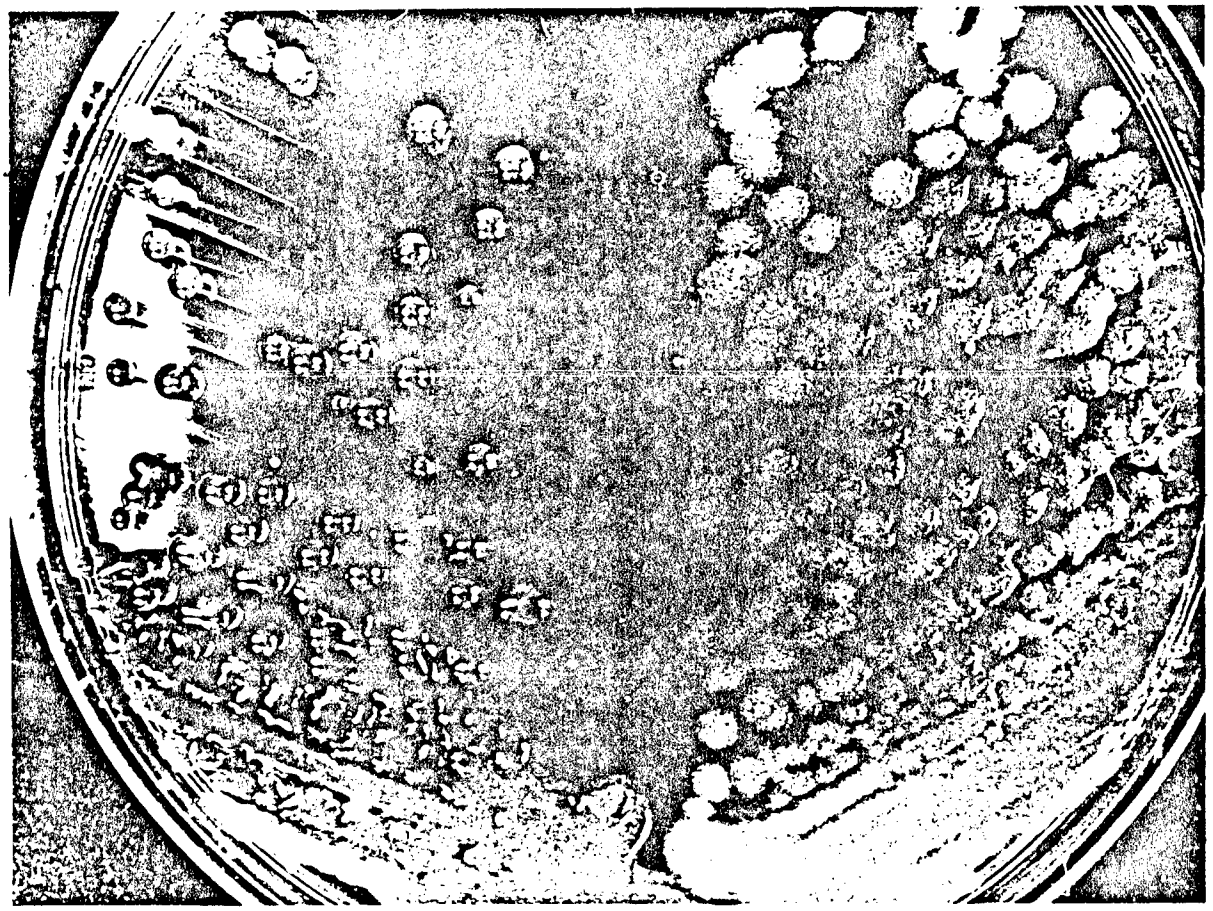


FIG. 5. Colonies of B. anthracis Pasteur strain grown on bicarbonate agar in 20% CO_2. Left, strain 6602 wild type. Right, 6602 R1 (rough variant of 6602).

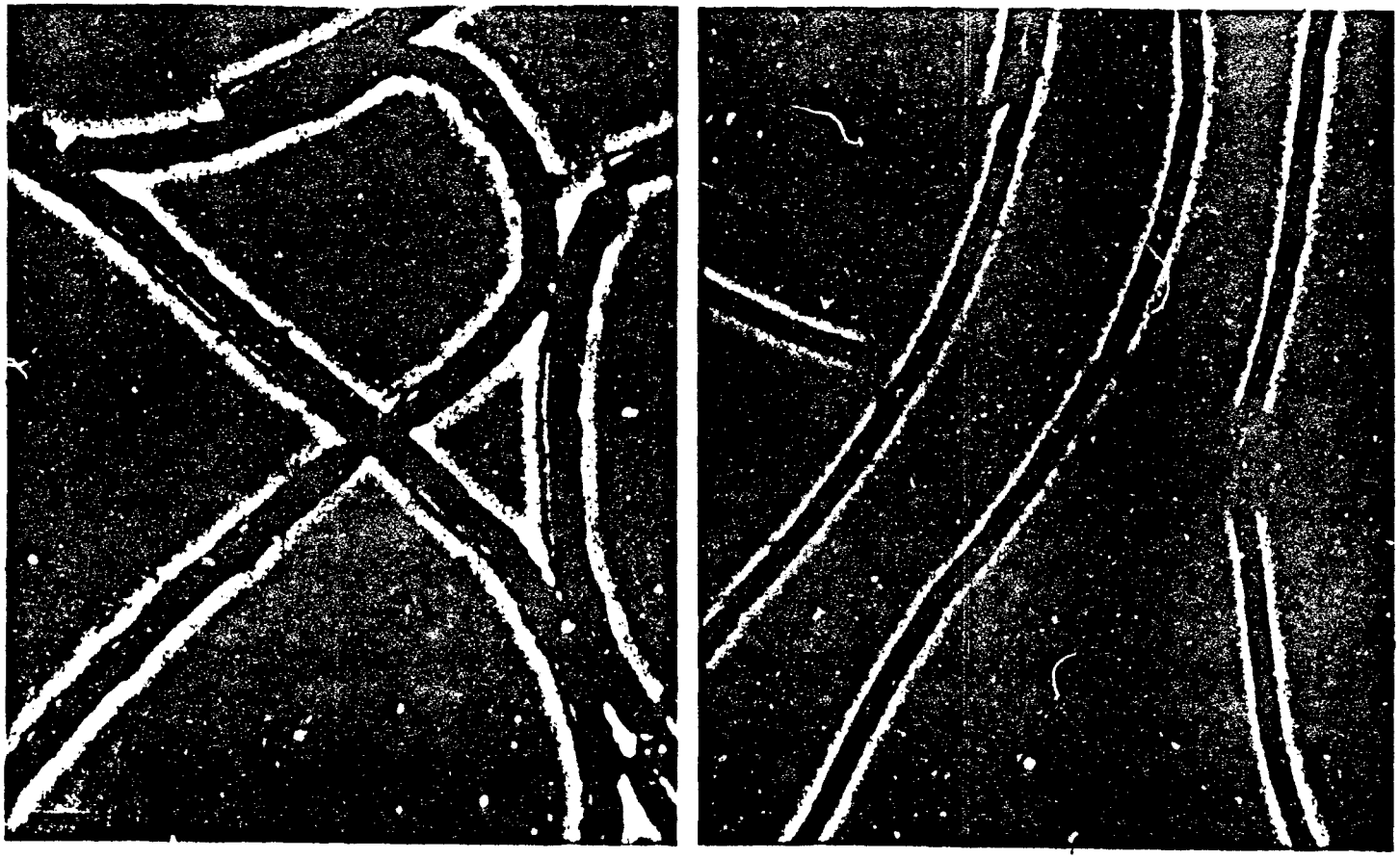


FIG. 6. Phase-contrast photomicrograph of cells of B. anthracis Pasteur strain grown on bicarbonate agar in 20% CO_2. Left, strain 6602 wild type. Right, 6602 R1 (rough variant of 6602).

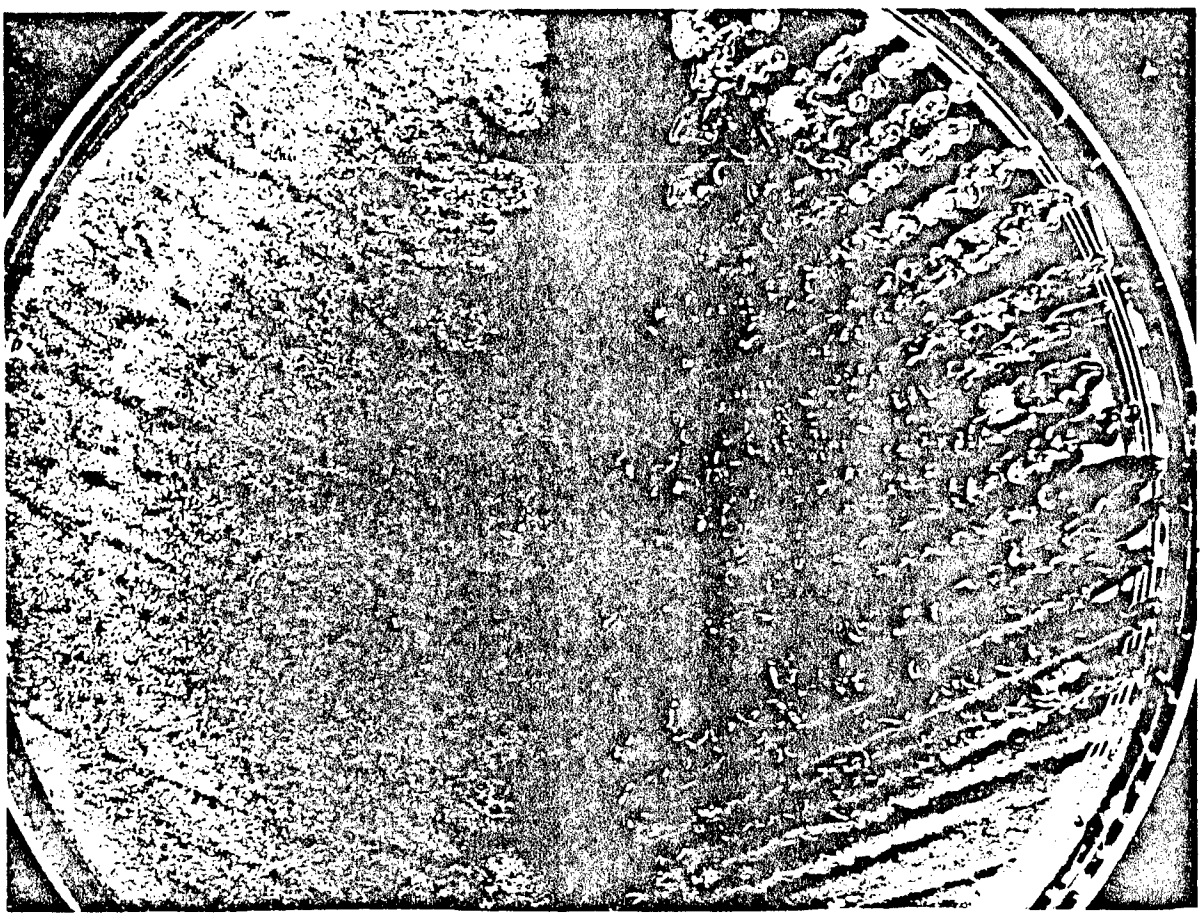


FIG. 7. Colonies of B. cereus grown on bicarbonate agar in 20% CO_2. Left, B. cereus 569 UM20-1. Right, B. cereus 569 UM20-1 tr49G-4, a Cap^+ transcipient carrying pXO2.

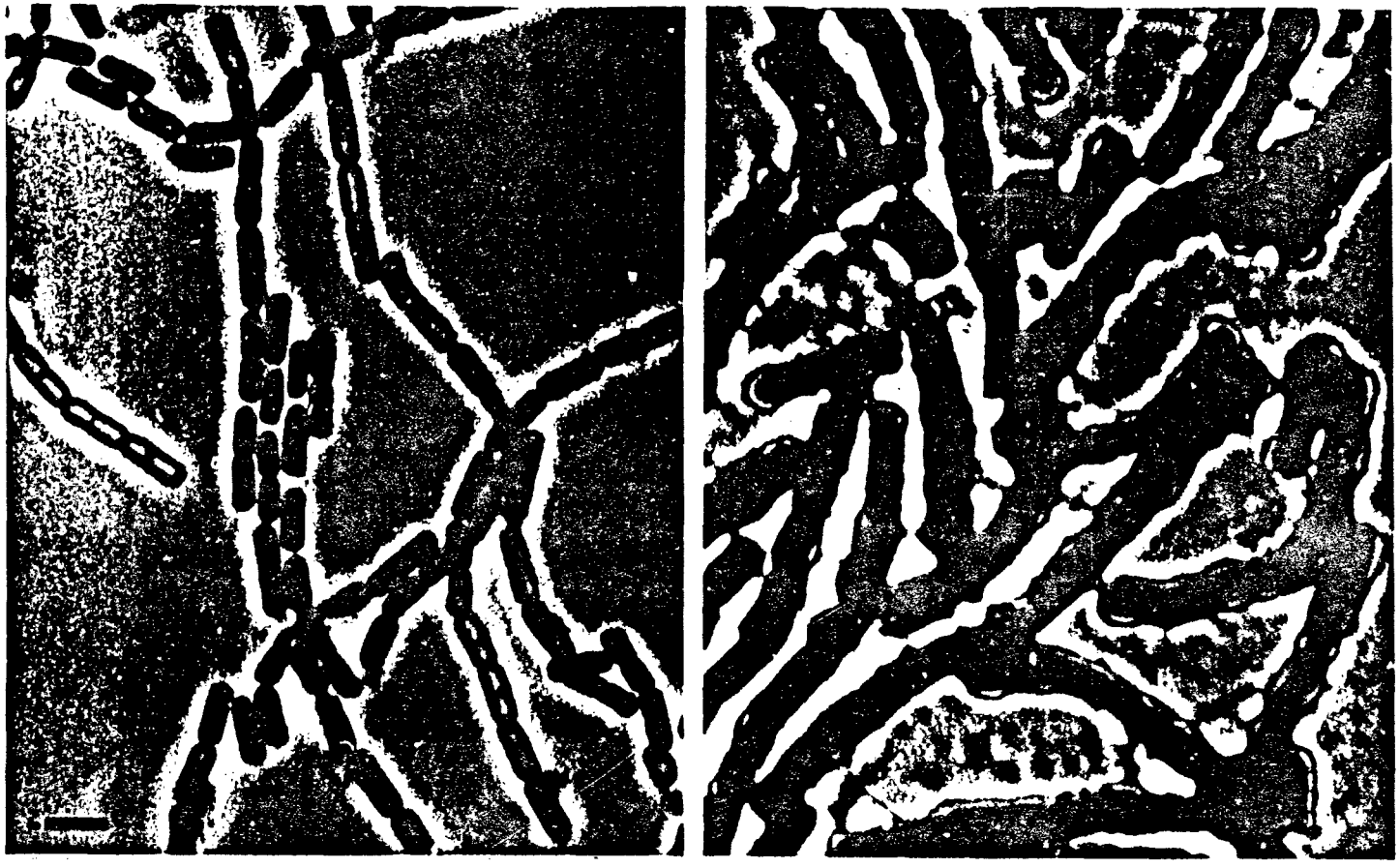


FIG. 8. Phase-contrast photomicrograph of cells of B. cereus grown on bicarbonate agar in 20% CO_2. Left, B. cereus 569 UM20-1 Cap^-. Right, B. cereus 569 UM20-1 tr49G-4, a Cap^+ transcipient carrying pXO2.

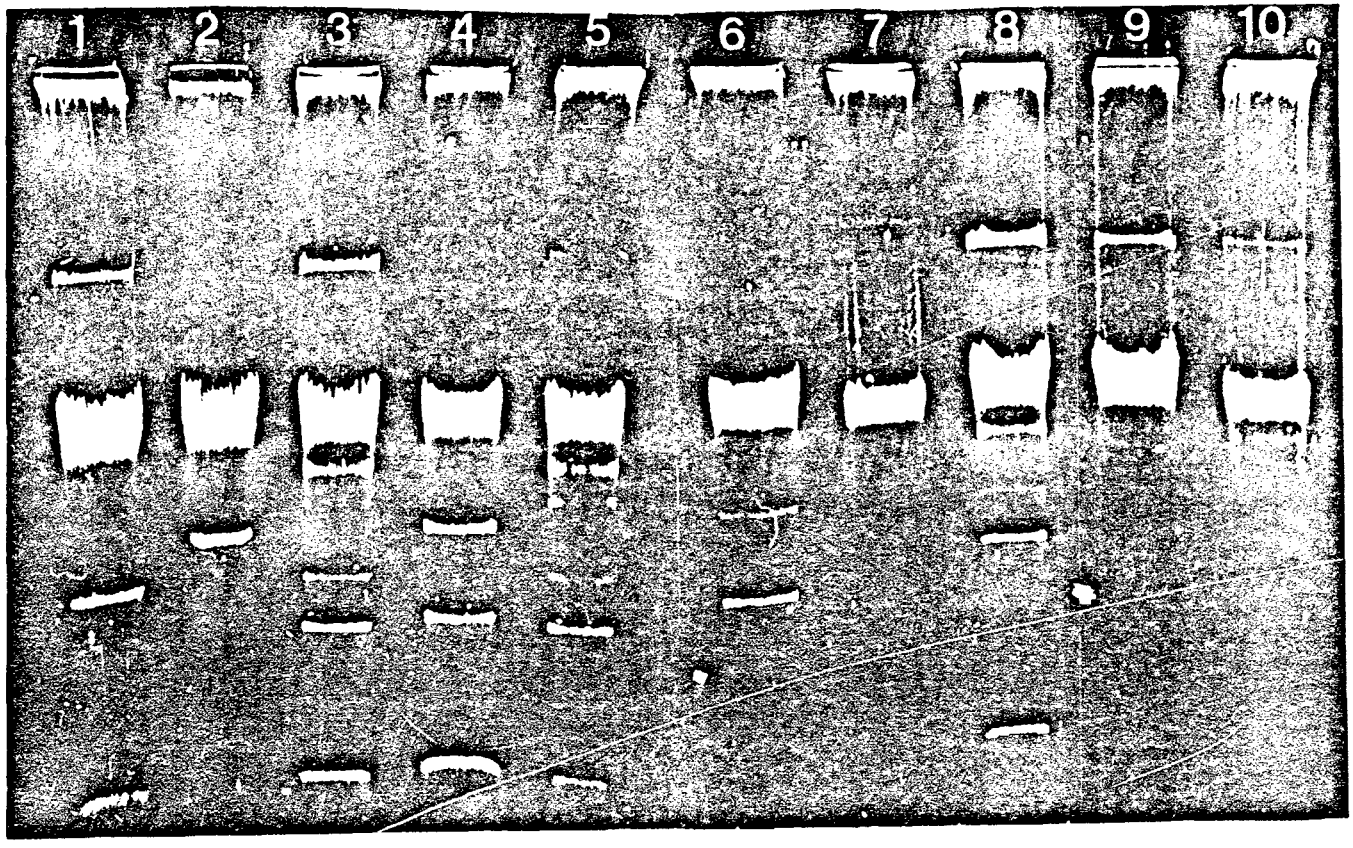


FIG. 9. Agarose gel electrophoresis of Cap^+ and Cap^- donors, recipients, and transcipients. Lane 1, B. anthracis 6602 tr172B-2 $Cap^+Cry^+Tc^r$; Lane 2, B. cereus 569 UM20-1; Lane 3, B. cereus 569 UM20-1 tr60G-6 $Cap^+Cry^+Tc^r$; Lane 4, B. cereus 569 UM20-1 tr60G-10 $Cap^+Cry^+Tc^r$; Lane 5, B. cereus 569 UM20-1 tr60G-11 $Cap^-Cry^+Tc^r$; Lane 6, B. cereus 569 UM20-1 tr60G-10 $Cap^+Cry^+Tc^r$ (same as lane 4); Lane 7, B. anthracis UM23C1-2 tr66G-1 $Cap^+Cry^+Tc^r$; Lane 8, B. cereus 569 UM20-1 tr49G-4 $Cap^+Cry^+Tc^r$; Lane 9, B. anthracis UM23C1-2 tr63G-10 $Cap^+Cry^+Tc^r$; Lane 10, B. anthracis UM23C1-2 tr63G-21 $Cap^-Cry^+Tc^r$.

ABSTRACTS OF PAPERS TO BE PRESENTED

Two papers will be presented at the March 1985 meeting of the American Society for Microbiology. Abstracts of the two papers are included here.

Interspecies Plasmid Transfer during *Bacillus* Matings

Laurie Battisti, B. D. Green, and C. B. Thorne

A mating system has been developed which promotes plasmid transfer among the three *Bacillus* species, *B. thuringiensis*, *B. anthracis*, and *B. cereus*. Transfer of the selectable tetracycline resistance plasmid, pBC16, and other resident plasmids from *B. thuringiensis* donors to *B. cereus* and *B. anthracis* recipients occurred during mixed incubation in broth cultures. Plasmid transfer was resistant to DNase, and cell-free filtrates of donors were ineffective. Two plasmids, pXO11 and pXO12 from *B. thuringiensis* subsp. *thuringiensis*, were responsible for plasmid mobilization. Transcipients harboring either pXO11 or pXO12 were, in turn, effective donors. Phase microscopy revealed that transcipients inheriting pXO12 had also acquired the ability to produce parasporal crystals (Cry^+) resembling those produced by *B. thuringiensis* donors, while those inheriting pXO11 were Cry^-. This plasmid exchange system has been useful in the analysis of genetic determinants residing on the *B. anthracis* toxin plasmid, pXO1 (112 megadaltons). *B. anthracis* transcipients inheriting pXO12 were capable of transferring pXO1 to genetically marked *B. anthracis* recipients that had been previously cured of pXO1. Thus, a mating system has beer. developed which promotes interspecies transfer of numerous *Bacillus* plasmids by a conjugation-like process.

Demonstration of a Capsule Plasmid in Bacillus anthracis

Brian D. Green, L. Battisti, and C. B. Thorne

Virulence of Bacillus anthracis is attributed to two factors: a tripartite exotoxin and a capsular polypeptide. It has been demonstrated previously that a 112-Mdal plasmid, pXO1, encodes the structural genes for toxin synthesis. We investigated whether a 60-Mdal plasmid, pXO2, present in virulent and avirulent strains capable of producing capsules, was involved in the synthesis of the capsule. B. anthracis Pasteur vaccine strain (ATCC 6602), an avirulent strain cured of pXO1 and unable to produce toxin, forms capsules when grown on agar containing bicarbonate and/or serum and incubated in 20% CO_2. Analysis of the plasmid content of rough noncapsulated variants of this strain revealed two classes: those which were cured of pXO2 and contained no detectable plasmid DNA, and those which still carried pXO2. Reversion to Cap^+ was demonstrable only in rough variants which harbored pXO2. By means of the Bacillus mating system in which plasmid transfer is mediated by the fertility plasmid, pXO12, pXO2 was transferred from B. anthracis 6602 to B. cereus 569R M20 Ant^- str-2. A B. cereus transcipient which acquired pXO2 and retained the Ant^- and Str^r markers of the recipient produced capsules under the same conditions required for capsule formation by B. anthracis. This confirms that pXO2 carries information required for the synthesis of capsules by B. anthracis.

PUBLICATIONS AND THESES

The following papers are in press or have been submitted for publication:

1. Thorne, C. B. Genetics of Bacillus anthracis. In Microbiology, 1985. American Society for Microbiology. In press.

2. Green, B. D., L. Battisti, C. B. Thorne, and B. E. Ivins. Demonstration of a capsule plasmid in Bacillus anthracis. Submitted for publication.

3. Battisti, L., B. D. Green, and C. B. Thorne. A mating system for transfer of plasmids among Bacillus anthracis, B. cereus, and B. thuringiensis. Submitted for publication.

The following Ph. D. dissertation was written on research carried out under this contract:

Robillard, Norman J. Changes associated with plasmid loss in Bacillus anthracis. Ph. D. Dissertation. University of Massachusetts. September 1984.

LITERATURE CITED

1. Barsomian, G. D., N. J. Robillard, and C. B. Thorne. 1984. Chromosomal mapping of Bacillus thuringiensis by transduction. J. Bacteriol. 157:746-750.
2. Clewell, D. B. 1981. Plasmids, drug resistance, and gene transfer in the genus Streptococcus. Microbiol. Rev. 45:409-436.
3. Debabov, V. G., R. R. Azizbekyan, O. I. Klebalina, V. V. D'yachenko, F. P. Galushka, and R. A. Belykh. 1977. Isolation and preliminary characteristics of extrachromosomal elements of Bacillus thuringiensis DNA. Genetika 13:496-501.
4. Gonzalez, J. M., Jr., B. J. Brown, and B. C. Carlton. 1982. Transfer of Bacillus thuringiensis plasmids coding for delta-endotoxin among strains of Bacillus thuringiensis and Bacillus cereus. Proc. Natl. Acad. Sci. U.S.A. 79:6951-6955.
5. Gonzalez, J. M., Jr., and B. C. Carlton. 1981. Plasmid transfer in Bacillus thuringiensis, p. 85-95. In U. N. Streips, S. H. Goodgal, W. R. Guild, and G. A. Wilson (ed.), Genetic exchange: a celebration and a new generation. Marcel Dekker, New York.
6. Gonzalez, J. M., Jr., and B. C. Carlton. 1984. A large transmissible plasmid is required for crystal toxin production in Bacillus thuringiensis variety israelensis. Plasmid 11:28-38.
7. Gonzalez, J. M., Jr., H. T. Dulmage, and B. C. Carlton. 1981. Correlation between specific plasmids and delta-endotoxin production in Bacillus thuringiensis. Plasmid 5:351-365.
8. Held, G. A., L. A. Bulla, Jr., E. Farrari, J. Hoch, A. I. Aronson, and S. A. Minnich. 1982. Cloning and localization of the lepidopteran protoxin gene of Bacillus thuringiensis subsp. kurstaki. Proc. Natl. Acad. Sci. U.S.A. 79:6065-6069.
9. Kado, C. I., and S. -T. Liu. 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. 145:1356-1373.
10. Klier, A., F. Fargette, J. Ribier, and G. Rapoport. 1982. Cloning and expression of the crystal protein genes from Bacillus thuringiensis strain berliner 1715. EMBO J. 1:791-799.

11. Kronstad, J. W., H. E. Schnepf, and H. R. Whiteley. 1983. Diversity of locations for Bacillus thuringiensis crystal protein genes. J. Bacteriol. 154:419-428.
12. McCloy, E. W. 1951. Studies on a lysogenic Bacillus strain. I. A bacteriophage specific for Bacillus anthracis. J. Hyg. Camb. 49:114-125.
13. Meynell, E. W. 1963. Reverting and non-reverting rough variants of Bacillus anthracis. J. Gen. Microbiol. 32:55-60.
14. Mikesell, P., B. E. Ivins, J. D. Ristroph, and T. M. Dreier. 1983. Evidence for plasmid-mediated toxin production in Bacillus anthracis. Infect. Immun. 39:371-376.
15. Minnich, S. A., and A. I. Aronson. 1984. Regulation of protoxin synthesis in Bacillus thuringiensis. J. Bacteriol. 158:447-454.
16. Perlak, F. J., C. L. Mendelsohn, and C. B. Thorne. 1979. Converting bacteriophage for sporulation and crystal formation in Bacillus thuringiensis. J. Bacteriol. 140:699-706.
17. Ristroph, J. D., and B. E. Ivins. 1983. Elaboration of Bacillus anthracis antigens in a new defined culture medium. Infect. Immun. 39:483-486.
18. Robillard, Norman J. 1984. Changes associated with plasmid loss in Bacillus anthracis. Ph. D. Dissertation. University of Massachusetts.
19. Schnepf, H. E., and H. R. Whiteley. 1981. Cloning and expression of the Bacillus thuringiensis crystal protein gene in Escherichia coli. Proc. Natl. Acad. Sci. U.S.A. 78:2893-2897.
20. Stahly, D. P., D. W. Dingman, L. A. Bulla, Jr., and A. I. Aronson. 1978. Possible origin and function of the parasporal crystals in Bacillus thuringiensis. Biochem. Biophys. Res. Commun. 84:581-588.
21. Sterne, M. Variation in Bacillus anthracis. Onderstepoort J. Vet. Sci. Animal Ind. 8:271-349(1937).
22. Thorne, C. B. 1960. Biochemical properties of virulent and avirulent strains of Bacillus anthracis. Ann. N. Y. Acad. Sci. 88:1024-1033.
23. Thorne, C. B. Transducing bacteriophage for Bacillus cereus. J. Virol. 2:657-662(1968).
24. Thorne, C. B., and F. C. Belton. An agar diffusion method for titrating Bacillus anthracis immunizing antigen and its application to a study of antigen production. J. Gen. Microbiol. 17:505-516(1957).
25. Vodkin, M. H., and S. H. Leppla. 1983. Cloning of the protective antigen gene of Bacillus anthracis. Cell 34:693-697.

DISTRIBUTION LIST

5 copies	Commander US Army Medical Research Institute of Infectious Diseases ATTN: SGRD-UIZ-E Fort Detrick Frederick, MD 21701-5011
4 copies	Commander US Army Medical Research and Development Command ATTN: SGRD-RMS Fort Detrick Frederick, MD 21701-5012
12 copies	Defense Technical Information Center (DTIC) ATTN: DTIC-DDAC Cameron Station Alexandria, VA 22304-6145
1 copy	Dean School of Medicine Uniformed Services University of the Health Sciences 4301 Jones Bridge Road Bethesda, MD 20814-4799
1 copy	Commandant Academy of Health Sciences, US Army ATTN: AHS-CDM Fort Sam Houston, TX 78234-6100